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1st Tiselius Symposium on Modern Biochemical Separation Techniques

Uppsala, June 13–17, 1977

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edited by

Michael Lederer

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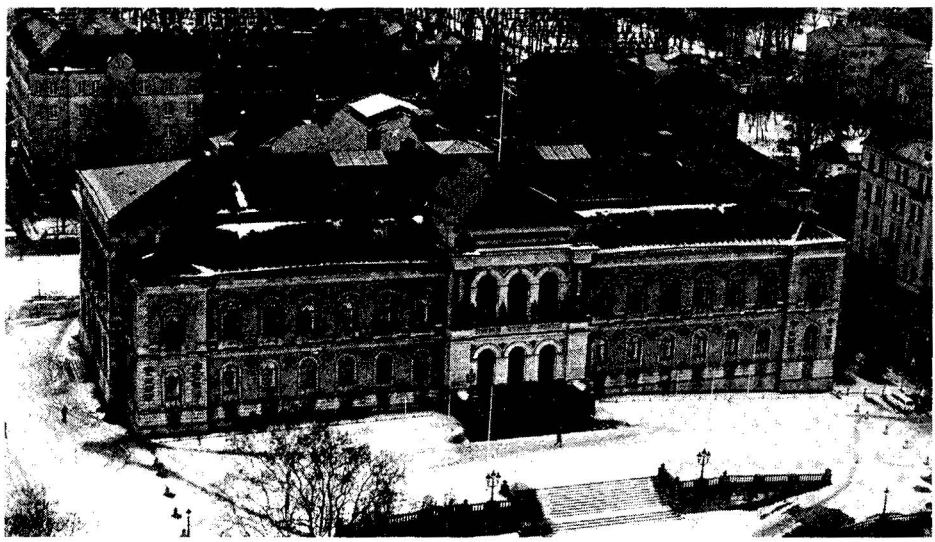
SPECIAL ISSUE



FIRST TISELIUS SYMPOSIUM ON MODERN BIOCHEMICAL SEPARATION TECHNIQUES

Uppsala (Sweden), June 13–17, 1977

Edited by

S. HJERTÉN
(Uppsala)

J. PORATH
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CHREV. 110T1

OPENING ADDRESS

Ladies and Gentlemen:

On behalf of the Organizing Committee, which consists of Professor Jerker Porath and myself from the Institute of Biochemistry and Dr. Jan-Christer Janson from Pharmacia Fine Chemicals, I wish to extend to all of you a hearty welcome to the first Tiselius Symposium. We hope that this symposium will lead to the development of new separation methods and the refinement of existing methods. If we succeed in this, then we shall have succeeded in honouring the memory of Arne Tiselius. As all of us who were acquainted with him well know, he was convinced that progress in biochemistry goes hand-in-hand with the development of new methods and apparatus.

Shortly after his death in 1971, the Tiselius Memorial Fund was created*. To the many among you participants who have personally contributed to this fund we extend our deepest thanks. According to the charter, "this fund in memory of Arne Tiselius should further the two subjects with which he was especially concerned, namely biochemical separation methodology and the positive and negative effects of scientific research on society and its future".

This, the first Tiselius Symposium, is naturally somewhat experimental, and we therefore welcome your opinions and suggestions regarding its organization. We were uncertain as to how the symposium should be organized so as to be as effective and rewarding as possible. We originally intended that none of the lectures should be published, with the thought that this might encourage the participants to discuss more freely new ideas and approaches that may not yet be solidly backed up by laboratory results. However, there have been many requests that the proceedings should be published and this will be arranged for all of you who so wish. But we do hope that this will not inhibit our discussions, which should be a vital part of this symposium. Bold and unconventional suggestions are therefore very welcome. The intended function of the lectures is to introduce topics and to stimulate exchange of views and not simply to present facts and results.

Tiselius often aired his conviction that scientific activity should be conducted in a tranquil atmosphere, for it is only then that a researcher can concentrate all of his attention on his project and achieve his finest work. We on the Organizing Committee therefore hope that this Symposium can proceed in a relaxed spirit. We have attempted to foster informality by inviting relatively few participants. Unhampered by the presence of a large audience the participants should feel free to talk about what occupies their minds today and what they intend to try in the near future in addition to what they have already done. The informal and intimate character of small symposia such as this tends to stimulate frank and therefore fruitful discussions.

* Subscriptions may still be remitted to Arne Tiselius Commemorative Fund, Uplandsbanken, Uppsala, Sweden (Account No. 1801-31-840-56).

Small symposia also have the advantage that the time schedule need not be strictly followed but can be adjusted according to the interest in the discussion after each presentation. Although our knowledge about different separation methods has increased greatly since the time of Tiselius' pioneering work on electrophoresis and chromatography, the theoretical treatment of separation methods lags far behind that of many other methods in chemistry, biochemistry and physics. The general discussions and in particular the round-table discussion (led by Prof. Porath) at this symposium would be especially valuable if they develop so as to convey to the audience where the limits of our knowledge of separation methods lie and where there are gaps of ignorance to be spanned. By focusing attention on these problem areas we might challenge sharp young minds to grapple with them and to carry our understanding further.

In this symposium we shall be particularly concerned with recently discovered separation methods and with outlines of methods that can be expected to emerge in the near future. I also hope that in the course of small group discussions we can devote a little time to some already familiar methods, especially with regard to how and under what conditions they were discovered. Probably every separation method has its own story and perhaps no useful generalizations regarding the discovery of separation methods can be made. However, if one considers the research atmosphere in which each new method was created I believe one will find common factors. It also ought to be interesting to discuss in small groups the importance of the inspirational milieu that often surrounds a great master. Perhaps such an aura, together with that which exists in institutions which have a tradition of high achievement in a particular area, is the best fertilizer for the germination of "the prepared mind", which is an essential factor in all research. The proposed small group discussions on the dependence of research achievement on the research environment might provide answers to this and other questions regarding the optimization of research activity, including the development of new separation methods.

Finally, we extend our warmest thanks to Pharmacia Fine Chemicals for generous economic support of this symposium and for help with many details of the arrangements.

STELLAN HJERTÉN

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CHREV. 110T12

CHROMATOGRAPHIC PROCEDURES THAT ARE PROVING USEFUL IN RESEARCH ON RIBONUCLEASES

STANFORD MOORE
The Rockefeller University, New York, N.Y. 10021 (U.S.A.)

CONTENTS

1. INTRODUCTION

The organizers of this symposium, and most of the members of this audience, have special memories of Arne Tiselius. I recall that on my first trip to Europe, in 1949, Uppsala was a key city on my itinerary. A young American on his initial journey overseas might be expected to think first of the major capitals in this part of the world; yet my thoughts turned especially toward the scholarly town of Uppsala and the University which it hosts. The presence of Arne Tiselius and the contributions that he was making to the methods of chromatography in those years was the source of my affinity for Uppsala. I came to consult him in reference to the experiments that William Stein and I were conducting at The Rockefeller Institute on the chromatography of amino acids, peptides, and proteins. A conversation with Arne Tiselius in Uppsala or in New York was always most stimulating to us, both personally and in terms of the roles of separation methods in biochemistry and the principles from which they could evolve. The spirit of this conference in Sweden stems from his leadership. The rate of progress in the development of separation techniques in the past thirty years has exceeded all expectations and a number of the innovations have come from the laboratories of the Institute of Biochemistry in Uppsala.

2. CHROMATOGRAPHIC PROCEDURES

Methods are a means to an end, not an end in themselves, and the main theme of my specific contribution this morning will be to give a few examples of the ways in which some of the current procedures for the separation of proteins are helping us to learn more about ribonucleases. In the interest of simplicity I will start with the elegantly simple technique of gel filtration which has rapidly grown into one of the most widely used methods in protein chemistry. It was during my visits to Uppsala in the 1950s that I first learned of the invention of gel filtration and Sephadexes by Porath and Flodin[1]. We are among the many who promptly found that the method

could be extremely useful. In the special journal volume dedicated to Arne Tiselius in 1962, Crestfield *et al.*[2] had the opportunity to describe (Fig. 1) the fractionation by gel filtration of the aggregates formed when bovine pancreatic ribonuclease is lyophilized from 50% acetic acid. The main product was a dissociable dimer of molecular weight about 28,000. Since then, renewed interest in dimers of ribonuclease has grown from the researches of Leone and D'Alessio and their colleagues[3,4] on a ribonuclease in bovine seminal plasma which is homologous in primary structure to the pancreatic enzyme of the same species but is a dimer cross-linked by two –S–S– bonds; Libonati and Floridi[5] found that the dimeric enzyme is more active toward double-stranded RNAs than is the pancreatic monomer. This observation prompted us to study the cross-linking of the pancreatic monomer by diimido esters with the aim of obtaining a stable dimeric derivative which would have increased activity toward poly A·poly U. Hartman and Wold[6] in 1967 and Wang *et al.*[7] in our laboratory used gel filtration of the same type illustrated in Fig. 1 to isolate the dimeric fraction from the reaction products. The most active dimeric derivative had about eight times the activity of the monomer toward the double-stranded substrate. But that is only a modest increase in activity. How might we gain a greater increase? D'Alessio *et al.*[4] and Libonati *et al.*[8,9] developed the thesis that the number of basic charges in a ribonuclease was the important variable in the action toward double-stranded substrates. Accordingly, Wang and Moore[10] undertook to use dimethyl suberimidate to cross-link ribonuclease to a polyamine, such as spermine, which has a special affinity for nucleic acid. In this instance, the gel filtration was used initially to remove any ribonuclease dimer from the desired spermine–ribonuclease fraction,

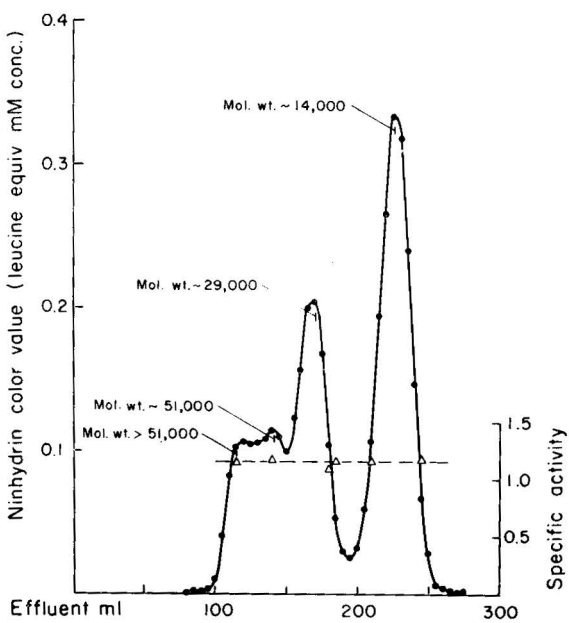

Fig. 1. Separation of ribonuclease A and its aggregates on a 2 × 143 cm column of Sephadex G-75 with 0.2 *M* sodium phosphate buffer (pH 6.47) as the eluent. The molecular weights were determined by ultracentrifugation. From ref. 2.

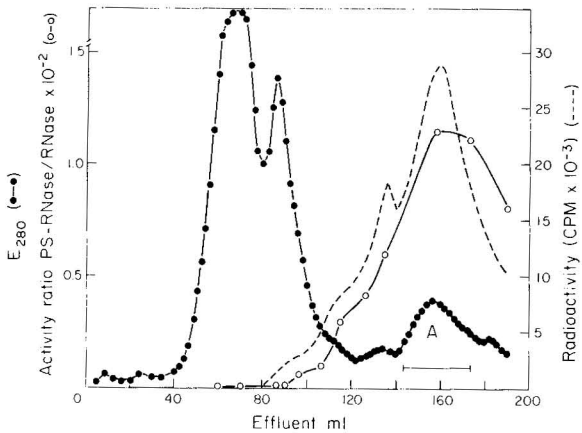

Fig. 2. Fractionation of polyspermine–RNases on carboxymethyl-Sephadex (C-50). Column, 0.9 × 60 cm. Elution was with a linear gradient of pH and NaCl concentration formed with 100 ml of 0.1 *M* phosphate buffer of pH 6.4 and 100 ml of 0.1 *M* phosphate buffer of pH 8.0, 0.7 *M* in NaCl. Activity was measured toward poly A·poly U. From ref. 7.

which was then submitted to ion-exchange chromatography on carboxymethyl-Sephadex (Fig. 2). The result was a product (from Zone A) which had an average of 8 spermine residues (measured by ^{14}C) per molecule of ribonuclease. The activity was about 100 times the activity of the native enzyme toward poly A·poly U and nearly 400 times the activity toward the hybrid substrate poly rU·poly dA. A natural double-stranded RNA from reovirus 3 could be completely converted to acid-soluble nucleotides in 10 min by polyspermine–ribonuclease.

In our early experiments on pancreatic ribonuclease, one of the approaches was to see whether we could lower the activity of the enzyme by specific derivatizations; in this way we plotted residues which were probably at or near the active site of the catalyst[11]. It is also informative to see whether you can make an enzyme more active than the native molecule. The cross-linking experiments with ribonuclease have been one step in this direction.

In the early days of our studies on the chromatography of proteins on ion exchangers, William Stein and I frequently discussed the theory of the process with Arne Tiselius. We were then seeking to obtain finite distribution coefficients which would permit a protein to be eluted by a single buffer. This objective was achieved in cooperation with Werner Hirs in 1951[12,13]; ribonuclease (Fig. 3) was eluted from the polymethacrylic acid resin Amberlite IRC-50 just as simply as was an amino acid from the same exchanger or from the sulfonated polystyrene resin Dowex 50 (ref. 15). But ribonuclease is a relatively small protein; Arne Tiselius discussed with us the mathematics of all-or-none adsorption that would be approached as multipoint attachment increased with larger proteins and the probable need for increases in pH and ionic strength for successful elution of large proteins from ion exchangers[16,17]. In 1954 he noted[16] that the rate of elution of polyvalent proteins will tend to change from an R_F value of zero to an R_F of one in a very narrow range of buffer concentration. In that article he also suggested that hydrophilic matrices might be preferable to hydrophobic ones (such as polymethacrylates) for many protein separations. The

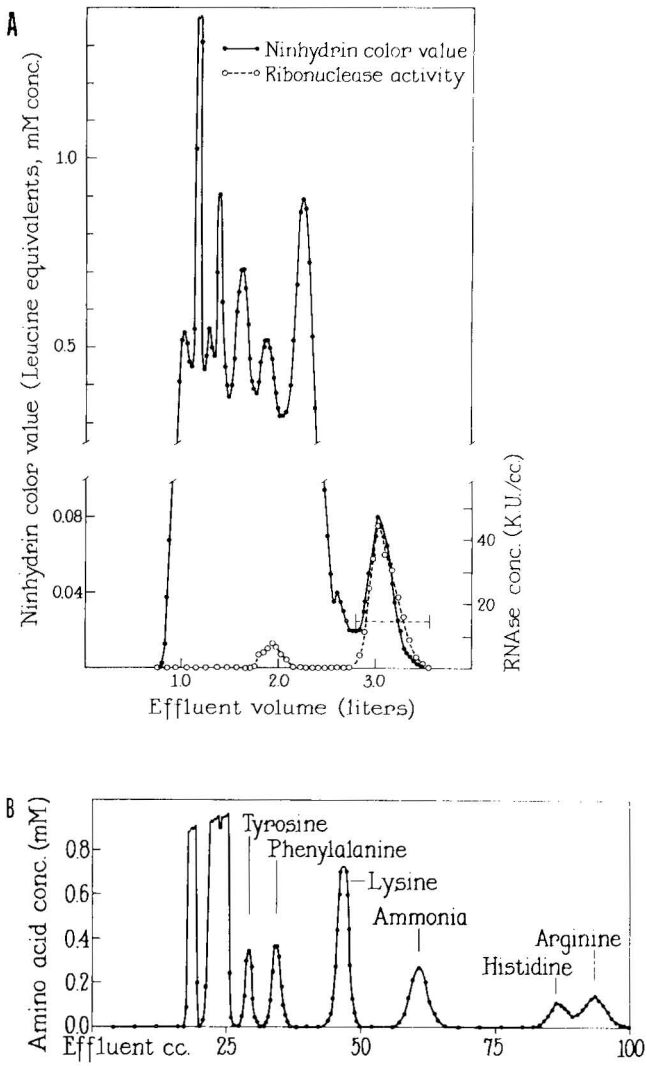

Fig. 3. (A) Chromatography of a 0.25 *M* sulfuric acid extract of comminuted bovine pancreas on the carboxylic acid resin IRC-50. Eluent, sodium phosphate buffer (0.2 *M*, pH 6.47); preparative column, 7.5 × 60 cm. (B) Chromatography of amino acids on the same resin (0.9 × 60 cm) with 0.5 *M* citrate buffer (pH 5.4). From ref. 13. The open circles give the ribonuclease activity; the smaller active peak (RNase B) was later characterized as a glycosylated derivative of RNase A by Plummer and Hirs[14].

exciting introduction by Peterson and Sober in 1956[18] of ion exchangers prepared from cellulose opened a whole new era in protein chromatography, in which exchangers based on the dextrans developed in Uppsala, such as the carboxymethyl-Sephadex used in Fig. 2, are among the most common reagents in protein chemistry.

Arne Tiselius[16], in the course of considering adsorbents for proteins, systematically explored hydrophilic gels of calcium phosphate in 1954 with the aim of making the adsorbent more generally useful. Ten years later, when we came to study

the homogeneity of pepsin[19] we noted that Tiselius *et al.*[20] in their report on the use of hydroxyapatite columns for a number of proteins, mentioned that in 1861 Brücke[21] reported that pepsin activity could be adsorbed and eluted from calcium phosphate. Brücke's research takes us back only about 100 years, which is not far relative to the 500 years of the University of Uppsala which we commemorate on this occasion. Chromatography of commercial pepsin on hydroxyapatite with increasing concentrations of phosphate gave us the chromatogram shown in Fig. 4; the extent of the heterogeneity prompted us to look for improved ways of preparing pepsin for structural study. A decade later, the same adsorbent was helpful in the experiments of Arabinda Guha in our laboratory on the purification of a phosphodiesterase from bovine brain (Fig. 5).

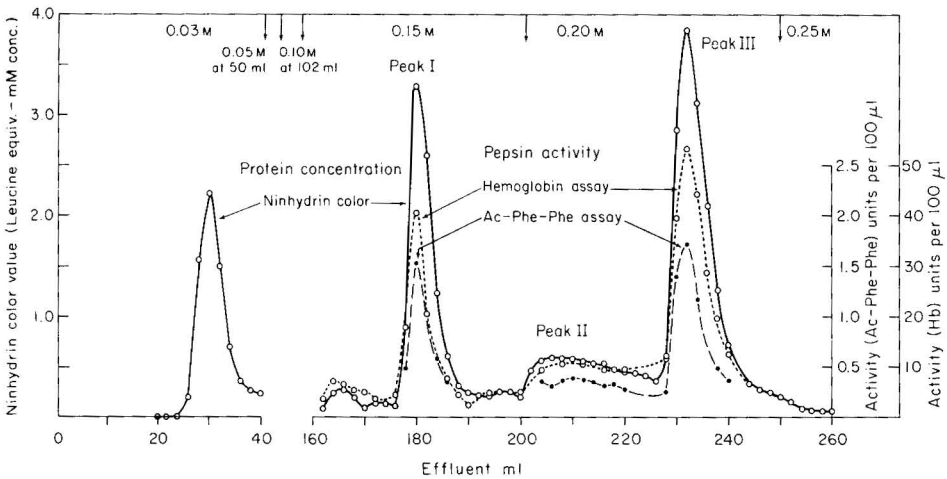

Fig. 4. Chromatography of commercial pepsin on a 0.9 × 40 cm column of hydroxyapatite. Phosphate buffers (pH 5.7) of increasing molarity were used as eluents. From ref. 19.

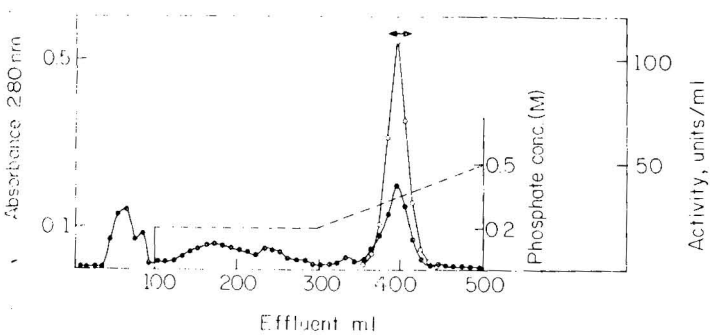

Fig. 5. Chromatography on hydroxyapatite of 2′,3′-cyclic nucleotidase from bovine brain; the sample was a partially purified fraction obtained by initial chromatography on carboxymethyl-Sephadex. The column (2 × 10 cm) was eluted with sodium phosphate buffer (pH 6.8) of increasing molarity. The open circles give the enzymic activity. From ref. 22.

The initial steps in the purification of 2′,3′-cyclic nucleotidase from brain[22] are special because the enzyme is initially insoluble. In order to utilize the potential resolving power of chromatography for the purification of proteins, the protein has to be brought into solution. Much of the progress in enzyme chemistry in recent decades has been with soluble proteins available in gram quantities. Advances in the current decade are being made with many enzymes that are present in much smaller amounts and that frequently are membrane-bound. It is this realization that is stimulating the scaling-down of the methods of amino acid analysis to the nanomole range[23] and that has prompted us to see whether we could arrive at methods that might be helpful in the isolation of some initially insoluble enzymes.

Detergents are frequently employed for solubilization; Drummond *et al.*[24], in their initial studies on 2′,3′-cyclic nucleotidase, used 6% Tween 20. But if there is a way to avoid detergents, we would prefer to be able to study the chemical and physical properties of an enzyme in plain aqueous solution. We have tried many combinations of conditions for the solubilization of the 2′,3′-cyclic nucleotidase in brain; one approach which is working is summarized in Table 1.

TABLE 1

STEPS IN THE SOLUBILIZATION AND PARTIAL PURIFICATION OF 2′,3′-CN 3′-ASE (FROM REF. 22)

The starting material was 20 g of an acetone powder prepared from about 100 g of bovine brain white matter.

Step	*Total activity (units)*	*Specific activity (units/mg protein)*	*Recovery (%)*
1. Initial extract (1 *M* in GuCl*) after centrifugation	12 000	2.5	100
2. Supernatant (0.2 *M* in GuCl*) after 5-fold dilution of (1)	9600	2.5	80
3. Precipitate obtained by addition of $(NH_4)_2SO_4$	9000	12.6	75
4. Carboxymethyl–Sephadex chromatography	3200	106	28
5. Hydroxyapatite chromatography	2500	508	21

* Guanidinium chloride.

The starting product in this instance is white matter, since this tissue is richest in the enzyme. Investigators who have studied the isolation of myelin find that the 2′,3′-cyclic nucleotidase is most closely associated with the myelin fraction, which is unusual, because most enzymic activities in myelin are very low. An acetone powder from bovine white matter is homogenized with 1 *M* guanidinium chloride at pH 6; the solution is also 1 m*M* in dithiothreitol and EDTA. More than half, but not all of the enzyme is thereby solubilized. If, from this solubilized portion we dialyze out the guanidinium chloride in one operation, the enzyme reprecipitates, and we are back where we started. But, if we carry out a fractional precipitation, a sort of "ammonium sulfate fractionation" in reverse, by reducing the guanidinium chloride concentration stepwise, precipitates are obtained at 0.8 *M*, 0.6 *M*, etc. The enzyme stays in solution down to 0.2 *M*, and if the precipitate obtained at this guanidinium chloride concentration is spun off, the remaining solution can then be dialyzed completely free of guanidinium chloride without precipitation of the enzyme. The test for solubility is

retention of activity in the supernatant solution after centrifugation at 100,000 *g* for 4 h. We hope that this approach may be useful with some other membrane-bound proteins.

Our interpretation of this experiment is that the fractionation has separated the enzyme from a component in the extract with which the enzyme has a tendency to aggregate. The enzyme obtained at this stage can be concentrated by precipitation with $(NH_4)_2SO_4$ and redissolved for gel filtration and ion-exchange chromatography. After passage over carboxymethyl-Sephadex and hydroxyapatite, a 200-fold purification has been achieved.

The final example of methodology that we will discuss today is the application of affinity chromatography in our laboratory to the isolation of a ribonuclease inhibitor from human placenta by Blackburn and Wilson[25]. The presence in mammalian tissues of an inhibitor of neutral ribonuclease of the pancreatic type was first studied by Roth[26]. The biological connotations of the inhibitor's presence have recently grown in interest as it has become apparent that the ability of such an inhibitor to repress ribonuclease action may have a role in preserving the integrity of mRNA and polyribosomes[27] and hence facilitate protein synthesis and cell proliferation both *in vitro* and *in vivo*. The technique of affinity chromatography is ideally suited to the search in a homogenate for an inhibitor of an enzyme that can be attached to a suitable support. In this instance, pancreatic ribonuclease has been coupled to Sepharose 4B by the cyanogen bromide method pioneered by Porath and Axén[28]. The coupling with ribonuclease has been most complete when the cyanogen bromide (100 mg/ml settled bed) was dissolved (*cf.* ref. 29) in a minimum volume of a water-miscible organic solvent [we used N,N-dimethylformamide, approx. 1:1 (w/v)] and the solution was added dropwise to the stirred Sepharose suspension. When 50 mg of RNase A were coupled to 40 ml of CNBr-activated Sepharose, the final product contained about 1 mg of RNase per ml of settled Sepharose and did not shed detectable enzyme activity in the eluents, provided Tris-buffer was not used. The deleterious effect of Tris and other amino-containing buffers on the stability of ligands attached to CNBr-activated Sepharose has been reported by Wilchek *et al.*[30] and by Tesser *et al.*[31].

Human placentas obtained within 30 min of normal term delivery were homogenized in buffered 0.25 *M* sucrose, 5 m*M* in dithiothreitol and 1 m*M* in EDTA. A 35–50% ammonium sulfate precipitate was prepared, the ammonium sulfate was removed by dialysis, and a preliminary fractionation was conducted on DEAE-cellulose. The inhibitor is an acidic protein which is retarded on the exchanger at pH 7.5. The fraction containing the inhibitor was equilibrated with 45 m*M* phosphate buffer at pH 6.4 and applied to a column of RNase–Sepharose. The inhibitor was strongly bound; elution was accomplished (Fig. 6) by decreasing the affinity of the inhibitor for the enzyme by lowering the pH to 5.0, increasing the salt concentration to 3.0 *M* in NaCl, and including glycerol (15%, v/v) to increase the stability of the protein. The inhibitor thus eluted gave a single band[25] upon SDS-gel electrophoresis. The overall recovery was 45% (Table 2). The retention of activity was dependent upon the use of both dithiothreitol and glycerol.

The inhibitor is isoionic at pH 4.6, has a molecular weight of about 51,000, and forms a 1:1 molecular complex with RNase A with a K_i of $3 \cdot 10^{-10}$ *M*. Affinity chromatography has thus made available the pure inhibitor in a form that can be used

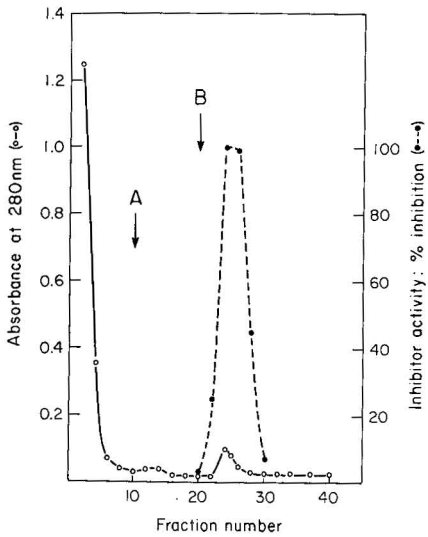

Fig. 6. Affinity chromatography of the ribonuclease inhibitor from human placenta on RNase–Sepharose (settled bed 8 ml). The sample was a partially purified fraction from a DEAE-cellulose column. The initial eluent was 45 m*M* phosphate buffer (pH 6.4). At A the buffer was made 0.5 *M* in NaCl. At B elution was initiated with 20 m*M* acetate buffer, pH 5.0, 3 *M* in NaCl and 15% (v/v) in glycerol. From ref. 25.

TABLE 2

PURIFICATION OF RNASE INHIBITOR FROM HUMAN TERM PLACENTA (FROM REF. 25)

Step	*Total protein (mg)*	*Total inhibitor (units)*	*Specific activity (units/mg)*	*Recovery of activity (%)*
1. Initial extract	16,000	400,000	25	100
2. 35–50% ammonium sulfate precipitate	2,327	320,000	137.5	80
3. 48,000 *g*, 1 h	1,646	320,000	194	80
4. DEAE-cellulose chromatography	308	240,000	780	60
5. Sepharose–RNase A affinity chromatography	1.8	180,000	100,000	45

in studies of its interaction with cellular RNase and its effects on RNase activity in *in vitro* translation and transcription studies.

These comments have illustrated some of the ways in which chromatographic methods, a number of which originated in Uppsala, have contributed to the isolation of proteins in our laboratory. The program of the First Tiselius Symposium includes reports on many experimental techniques that will have a direct bearing on the progress of biochemistry. The epoch in biochemistry covering the past thirty years has been one of great growth in our science. Methodology has had a key role in this process. The invention of new methods and new instruments and the cooperation of academic and industrial scientists in making supplies and equipment available to the

researcher have contributed to the practice and the art of separating molecules. The literature today emphasizes the accelerated rate of discovery of proteins and of elucidation of their structures. It is important to realize how much remains to be done. Protein chemistry today is in a stage of development that bears some similarity to the state of organic chemistry at the turn of the century. At that time there was great activity in expanding the volumes of Beilstein with characterizations of the myriad small organic compounds conceivable by man and nature. Today, in the polypeptide field, the atlas of protein structures is one volume; in the year 2000 it will be an encyclopedia of many volumes. And there will be a companion series on nucleic acids. Knowledge of macromolecular architecture is a prerequisite for fundamental understanding of the intricate synergisms of living systems and separation methods which we will discuss this week will have an increasing role in the accumulation of such information.

3. SUMMARY

Examples are given of the uses of gel filtration, chromatography on calcium phosphate, ion-exchange chromatography, and affinity chromatography, in the course of researches on pancreatic ribonuclease, dimeric derivatives of the enzyme, the 2′,3′-cyclic nucleotide 3′-phosphohydrolase of brain, and the inhibitor of ribonuclease of the pancreatic type in the human placenta.

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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 $\int\psi_0\,\psi d\nu = 0$, *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

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

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, Λ 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

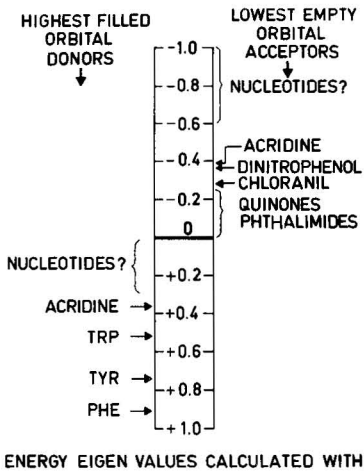


Fig. 1. Scale of energy eigen values (k_j values) obtained from approximate solution of the Schrödinger equation: $E_j = \alpha + k_j\beta$ where E_j is the electron energy in the j-orbital. α the coulombic integral and β the exchange integral. The scale has been drawn from data in ref. 3. A small k_j 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-

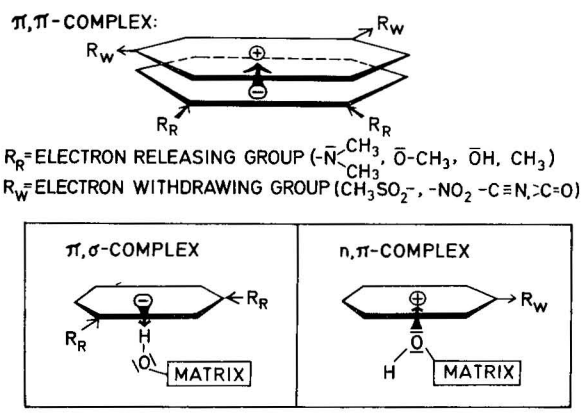


Fig. 2. Schematic representation of some types of complex that are involved in charge-transfer adsorption on polyhydroxylic gels.

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).

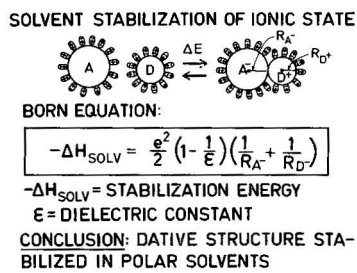


Fig. 3. Illustration of solvent interaction.

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.

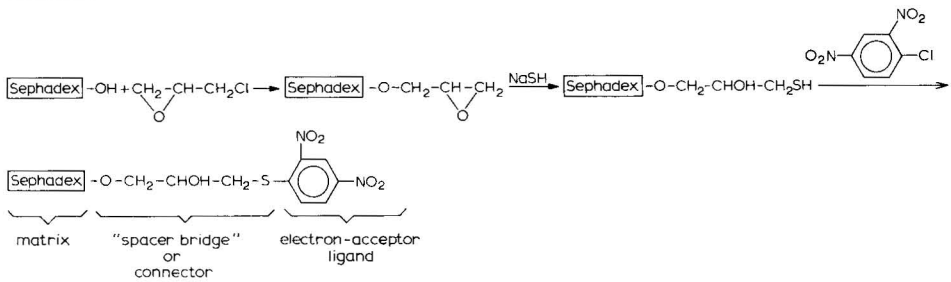
2.3. π,π complex-based adsorption

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.

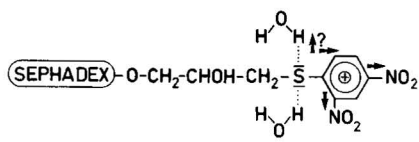
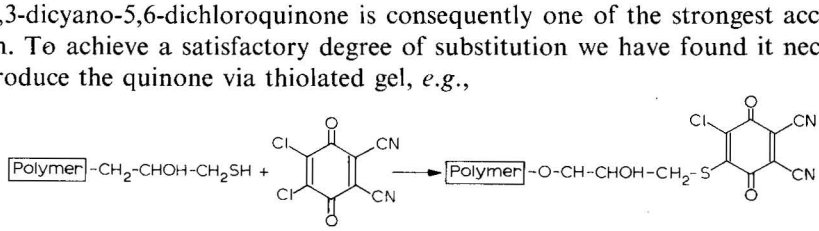


Fig. 4. Structure of DNP-S-Sephadex. Solvation is thought to explain the direction of electron withdrawal from the sulphur atom.

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.*, ΔH = 5–50 kJ/mole).

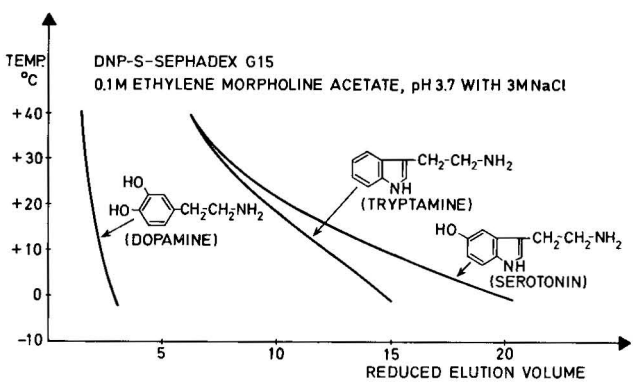


Fig. 5. Diagram showing how the adsorption of aromatics depends on the temperature. Separation is maximal at 0°.

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.

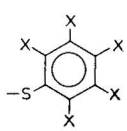
TABLE I

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

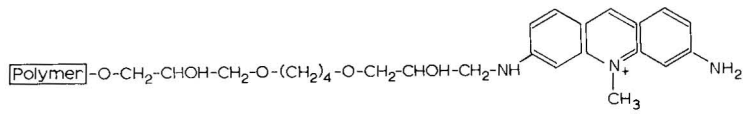
2.3.2. π-electron donor gels

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.

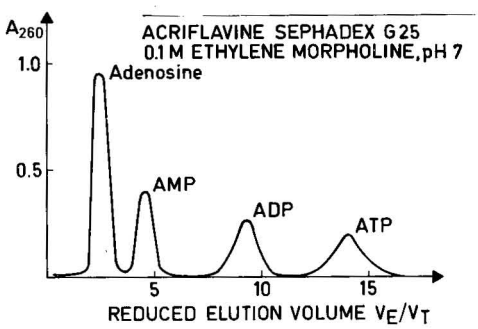


Fig. 6. Fractionation of adenosine and adenine ribonucleoside phosphates.

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

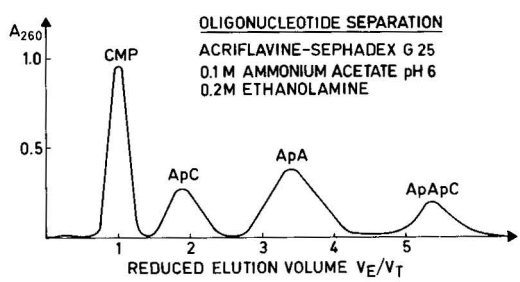
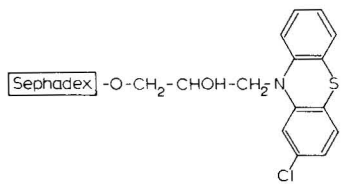


Fig. 7. Fractionation of some oligonucleotides on acriflavine-Sephadex under conditions where ionic adsorption ("ion exchange") is suppressed.

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:



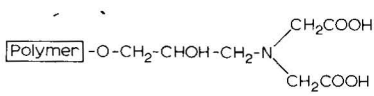
The coupling procedure has not yet been optimized, but the gels appear to be promising. They are only weakly coloured and show the expected affinities for nucleotides and other π-electron acceptor substances.

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.

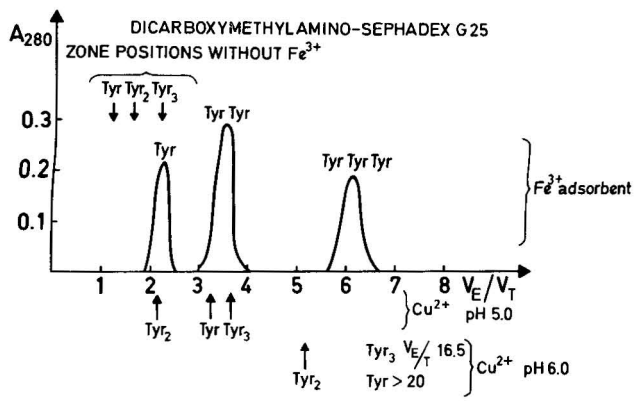


Fig. 8. Schematic diagram indicating the position in terms of V_E/V_T units of tyrosine and di- and trityrosines on Fe^{3+} and Cu^{2+} (lower part) loaded dicarboxymethylamino-Sephadex G-25. All chromatograms were run on the same column with and without the metal ion indicated. The chromatographic experiments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

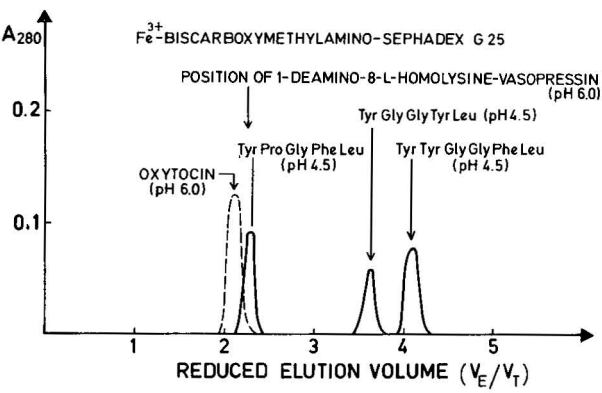
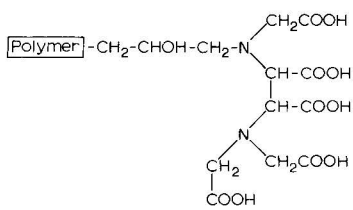


Fig. 9. Elution positions of some tyrosine-containing peptides on Fe^{3+}–chelate Sephadex G-25 in 0.1 *M* ammonium acetate, pH 5.0. Peptides containing a single tyrosine residue move faster than those containing two tyrosine residues per molecule.

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.

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CHREV. 110T15

USE OF THIOL–DISULPHIDE INTERCHANGE REACTIONS IN PREPARATIVE WORK ON PLASMA PROTEINS

C.-B. LAURELL

Department of Clinical Chemistry, University of Lund, Malmö General Hospital, S-214 01 Malmö (Sweden)

CONTENTS

1. INTRODUCTION

Thiol–disulphide interchange reactions with solid-phase (agarose gel beads)-linked thiol compounds (homocysteine, glutathione)[1,2] have been proposed as aids for immobilizing and stabilizing enzymes with labile SH groups and for fractionating proteins and peptides[3]. These SH–SS interchange reactions have been based on the mixed disulphides of the solid-phase-linked thiol after activation with 2,2′-dithiopyridine. This mixed disulphide renders exchange reactions with thiol compounds possible even below pH 7[4].

Our interest in SH–SS interchange reactions arose from clinical observations. On electrophoretic analysis of a myeloma serum, we observed an atypical occurrence of small protein bands in the inter-α-zone. They could not be explained by any myeloma proteins or by any free light chains. The atypical bands were identified as SS-linked complexes between κ-chains and three plasma proteins: prealbumin, albumin and α_1-antitrypsin (α_1AT)[5]. κ-Chains are known to have a reactive terminal cysteine. The albumin and α_1AT had formed complexes in the highest and similar concentrations. Prealbumin had given a series of complexes. This relation was unexpected as albumin was considered to be the dominating plasma protein with a reactive thiol group whereas each prealbumin subunit had been claimed to have a very unreactive thiol[6] and no cysteine had at that time been found in α_1AT. These statements on the content of reactive thiols in the proteins and their formation of SS-linked complexes *in vivo* were contradictory and prompted further studies.

2. EXPERIMENTAL AND RESULTS

The occurrence of complexes between κ-chains and albumin and α_1AT was explored in a few hundred filed myeloma sera with immunochemical methods. The

results suggested the conclusion that secretion of increased amounts of free κ-chains into blood regularly caused the formation of disulphide-linked complexes with α_1AT and albumin in similar proportions. The content of complexes in plasma showed a significant correlation ($r = 0.66$) to the amount of κ-chains secreted as estimated from the amount of κ-chains excreted with the urine[7]. The complexes could be formed *in vitro* on mixing of plasma and κ-chains. The yield of complexes was much enhanced if the κ-chains were added after their cysteine had formed a mixed disulphide with Ellman's reagent, 5,5'-dithiobis-(2-nitrobenzoate) (Nbs_2^{2-}). The complex formation was most rapid at about pH 8[8]. This suggested that the complex-forming plasma proteins had reactive thiol groups with p*K* values above 7.5. Of the other major plasma proteins, subfractions of IgA and β-lipoproteins also gave κ-chain complexes. The tendency for complex formation at biological pH was found to be 10–20 times greater for α_1AT and prealbumin than for albumin. This was a new property of α_1AT, which was shown[9] to depend on a single reactive cysteine in α_1AT.

One of the tasks of our research group was to develop methods for the isolation of native α_1AT without changing its electrophoretic microheterogeneity. Such α_1AT was available only in small amounts but was requested in large amounts for biological and chemical studies. The charge heterogeneity would not be altered in purification steps utilizing SH–SS interchange reactions. Therefore, we coupled monomeric κ-chains with Nbs-blocked terminal cysteine to cyanogen bromide-activated Sepharose. On slow passage of plasma (2 ml/ml of κ-Sepharose per minute) through such columns, about 50% of the retained proteins at pH 8.1 was α_1AT. Proteins bound through SS bridges could be stripped off efficiently by reduction with 0.02 *M* β-mercaptoethanol and the κ-chain column was ready for a new interchange cycle after reactivation of the κ-thiol with Nbs_2[10].

The SS bridges between κ-chains and the various plasma proteins varied slightly in their sensitivity to reduction. Fractionated elution was achieved by reversing the reaction used for the linkage (1) through application of a pH 8 buffer with a molar ratio of Nbs to Nbs_2 of 2:1. The free SH group of uncoupled α_1AT molecules immediately reacted with Nbs_2 (reaction 2):

$$\alpha_1AT(s^-) + Nb(ss)\kappa \rightleftharpoons \alpha_1AT(ss)\kappa + Nbs^- \quad (1)$$

$$\alpha_1AT(s^-) + Nbs_2 \rightleftharpoons \alpha_1AT(ss)Nb + Nbs^- \quad (2)$$

SH–SS interchange chromatography has been combined with antibody affinity chromatography to eliminate remaining impurities[10]. This isolation procedure has been automated by utilizing a series of pumps and valves. The flow system is governed by a punch strip and an electronic unit with a time constant of 10 min. When starting with the κ-chain in the Nbs_2 derivatized form, the sequence of the various steps of the fractionation cycle are as follows:

I	Washing off excess Nbs_2 from the κ-chain column with Tris buffer, pH 8.
II	Sample application, pH 8.
III	Washing off plasma with Tris buffer, pH 8.
IV	Washing off plasma with phosphate buffer, pH 5.5.

V Washing off plasma with Tris buffer, pH 8.
VI Uncoupling of SS-linked proteins with Nbs/Nbs_2 in Tris buffer, pH 8.
VII Reduction of the κ-chains with β-mercaptoethanol in Tris buffer, pH 8.
VIII Washing with Tris buffer, pH 8.
IX Reactivation of the κ-chains with Nbs_2 in Tris buffer, pH 8.

The eluate obtained during step VI is transfered to a column with Sepharose-linked antibodies to absorb the undesired proteins, while α_1AT passes. The antibody column is reactivated by passage of 3.5 *M* ammonium thiocyanate solution, which dissociates the antigen–antibody complexes. The production capacity of an automated system using 200-ml κ-chain and antibody columns is about 250 mg/day of α_1AT (more than 95% pure) from plasma in a yield of about 40%. So far the column capacity has remained roughly unaltered after about 50 cycles.

The SH–SS interchange reactions can be used as an efficient step for enrichment of some other plasma proteins with reactive thiols, *e.g.*, prealbumin[11].

Nbs_2-activated λ-chains showed much slower interchange reactions with plasma proteins than activated κ-chains with COOH-terminal cysteine. λ-Chains have their reactive cysteinyl in a penultimate position with a COOH-terminal serine, similar to Sepharose-linked glutathione with glycine as the COOH-terminal. However, glutathione bound more albumin in relation to α_1AT from plasma than the κ-chains. Spacer-linked cysteine was tested as a κ-chain substitute. Its efficiency in linking thiol proteins was high but its interchange pattern with various plasma protein resembled glutathione more than κ-chains. It is therefore apparent that the microchemical surrounding of the thiol group of the protein has a great influence on the interchange reaction[12].

It was less surprising that Nbs_2-activated β,β-dimethylcysteine (penicillamine) was relatively inactive to thiol proteins because of probable steric hindrance through the bulky methyl groups adjacent to the SS bridge. The protein thiol interchange reactions of Nbs-cysteine methyl ester showed a selectivity in protein linkage similar to κ-chains. The selectivity of the SH–SS reactions of Nbs-cysteinyl is thus little influenced by the presence of a carboxyl adjacent to the thiol group.

The highest recoveries from the interchange reactions were obtained when the plasma proteins had been freshly reduced before their application on the columns and the Sepharose-coupled thiol compound had been "activated" to a labile mixed disulphide with Nbs_2 or similar compounds[12]. Part of the plasma protein thiols probably occur as reduction sensitive mixed disulphides in plasma. One problem with thiol chromatography is that freshly reduced proteins give the highest protein retention but any remaining reducing substance in the loading solution immediately inactivates the κ-chains by cleaving their mixed disulphides. Excessive reduction of the proteins of the sample may create difficulties by cleaving internal SS bridges of some proteins that normally have no reactive thiol. This will cause contamination with undesired proteins. Irregular yields in thiol chromatography are usually secondary to varying degrees of reduction of the thiols of the protein in the samples.

The Nbs_2-activated forms of κ-chains and of other simpler thiol compounds are efficient in forming SS bridges with albumin, α_1AT and prealbumin, but these plasma proteins never form mutual complexes through SS linkage or dimers, with the exception of albumin. Affinity chromatography on solid-phase-linked thiol com-

TABLE 1

AMOUNT (mg) OF MAJOR PLASMA PROTEINS BOUND TO κ-CHAIN-, GLUTATHIONE- AND CYSTEINE-SEPHAROSE (50 ml) ON LOADING WITH 100 ml OF PLASMA

The SH–SS interchange reactions were performed at pH 8.1 with mildly reduced plasma and the matrix-bound thiol compounds in the form of mixed disulphides with 4,4′-dithiopyridine, 6,6′-dithiodinicotinic acid or 5,5′-dithiobis-(2-nitrobenzoate).

Protein	*4,4′-Dithiopyridine*			*6,6′-Dithiodinicotinic acid*			*5,5′-Dithiobis(2-nitrobenzoate)*		
	κ-Chain	*Glutathione*	*Cysteine*	*κ-Chain*	*Glutathione*	*Cysteine*	*κ-Chain*	*Glutathione*	*Cysteine*
Prealbumin	2	4	9	11	13	18	5	10	11
Albumin	2	10	58	14	121	160	5	23	68
α_1-Antitrypsin	56	48	71	63	55	62	65	58	59
IgA	5	19	6	30	23	12	6.8	20	8

pounds will be more or less efficient depending on the varying reactivity of the thiols of the proteins and of the interchanging mixed disulphide.

Nbs_2-activated thiols have been regularly used in a comparative study of the efficiency and selectivity of various Sepharose-linked compounds in thiol chromatography of plasma proteins[12]. Brocklehurst and Little[4] compared the reactions of some aromatic disulphides with papain and found differences in their influence on the SH–SS interchange reactions. Therefore, some commercially available, related aromatic disulphides were compared as "activators" in plasma fractionation experiments, namely 2,2′-dithiopyridine, 4,4′-dithiopyridine, 2,2′-dithio-5-nitropyridine, 6,6′-dithiodinicotinic acid and 5,5′-dithiobis-(2-nitrobenzoate). Three types of thiol columns, κ-chain-, cysteine- and glutathione-Sepharose, were used for each activator. In each experiment the three columns were simultaneously loaded from the same batch of plasma protein to avoid any effect of varying degree of reduction in the samples. Both the efficiency and the selectivity of the interchange reaction were found to vary. The extreme results were obtained with 6,6′-dithiodinicotinic acid and 2,2′-dithiopyridine, like 4,4′-dithiopyridine. The former gave a higher yield and a lower selectivity than the latter. The yields of four major plasma proteins using three matrix-bound thiol compounds in combination with three "activators" are given in Table 1. The selectivity pattern of each column was characteristic, like its relative efficiency, when using the same activator. The absolute amounts of protein obtained varied slightly in replicate experiments because a reproducible reduction of plasma proteins before

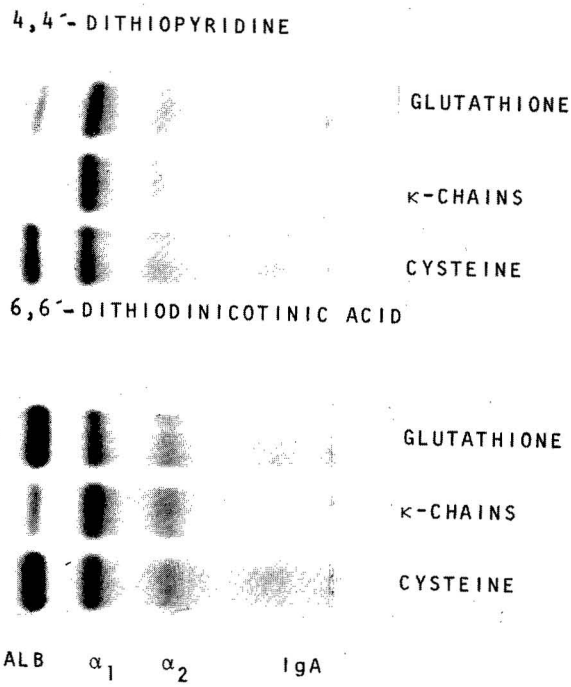

Fig. 1. Electrophoretic comparison of plasma proteins bound to glutathione-, κ-chain- and cysteine-Sepharose after activation with 4,4′-dithiopyridine and 6,6′-dithiodinicotinic acid.

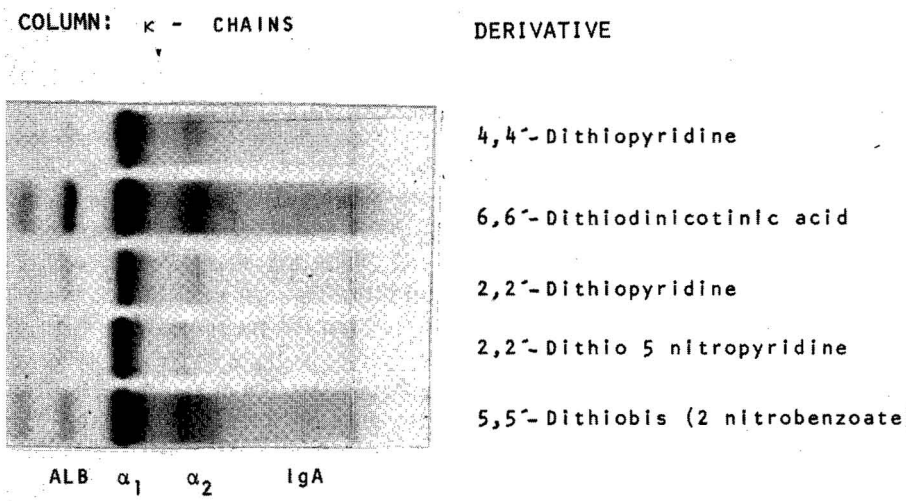

Fig. 2. Electrophoretic comparison of plasma proteins bound to κ-chain-Sepharose after activation with various aromatic thiol compounds.

loading the columns has not yet been developed. The qualitative difference (selectivity) in the SH–SS interchange reaction of plasma proteins is also apparent from Figs. 1 and 2, demonstrating the electrophoretic pattern of the proteins released from the three types of columns after elution with 0.02 *M* β-mercaptoethanol.

3. CONCLUSIONS

Both the activator and the matrix-bound thiol are of importance for obtaining maximal efficiency on fractionation by SH–SS interchange chromatography. The optimal combination of matrix-bound thiol and activator depends on the properties of the desired protein and cannot be predicted with our present knowledge of the interchange reactions.

4. ACKNOWLEDGEMENT

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5. SUMMARY

Thiol–disulphide interchange chromatography using Sepharose-linked thiol compounds is effective in the separation of plasma proteins. The efficiencies of Sepharose-linked κ-chains, glutathione and cysteine were compared using various aromatic disulphides as "activators". Both the activator and the matrix-bound thiol influence the efficiency and the selectivity of the fractionation of plasma proteins with SH–SS interchange chromatography.

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CHREV. 110T17

FRACTIONATION OF TRANSFER RIBONUCLEIC ACIDS BY CHROMATOGRAPHY ON NEUTRAL POLYSACCHARIDE MEDIA IN REVERSE SALT GRADIENTS

COLIN J. O. R. MORRIS

Department of Experimental Biochemistry, London Hospital Medical College at Queen Mary College, Mile End Rd., London E1 4NS (Great Britain)

CONTENTS

1. INTRODUCTION

Although about 80 transfer RNAs (tRNAs) have been isolated from a wide range of organisms (including an almost complete set for the 20 protein amino acids from *Escherichia coli*), their nucleotide sequences determined, and about 10 crystallized, a detailed three-dimensional structure is available for only one of these, yeast $tRNA^{Phe}$ (refs. 1 and 2). The other crystalline tRNA preparations have not yielded X-ray diffraction photographs of a quality adequate for high-resolution structural analysis. Various reasons have been advanced to account for this, but in my view a major contributory factor has been inadequate checking of the crystalline preparations for homogeneity. Several crystalline tRNA preparations which we have examined by high-resolution gel electrophoresis have shown gross inhomogeneity. Crystallinity is of little significance for homogeneity in the tRNA field, as the basic similarity in general structure among the different species makes co-crystallization very probable. In fact a crystalline preparation has been prepared from a mixed tRNA probably containing 30–50 individual species[3]. In the second place it is significant that yeast $tRNA^{Phe}$ is uniquely easy to purify, so that large amounts have been available for intensive purification and studies of the optimum conditions for crystallization.

The structural basis for the recognition of a specific tRNA by its cognate ligase remains unknown (for a review see ref. 4) and it is quite possible that different recognition mechanisms are operative in different tRNA-ligase systems. Although interchangeability at the ribosomal sites during protein synthesis imposes fairly rigid

constraints on the overall shapes and dimensions of the tRNA species, it now seems unlikely that much progress will be made with the tRNA-ligase recognition problem until the three dimensional structures of at least a majority of the set of tRNAs from one species are known.

As a first step towards this aim we have attempted to develop in this laboratory a general scheme for the isolation of a number of highly purified tRNA species from a mixed preparation made from yeast by the method of Holley[5]. This extract is very heterogeneous, and probably contains 50–60 individual species as well as partially degraded molecules formed during extraction. Fig. 1 shows a two-dimensional gel electrophoresis of a mixed tRNA preparation. At least 40 individual spots may be seen, many of which may be complex, while about 25 constitute major species.

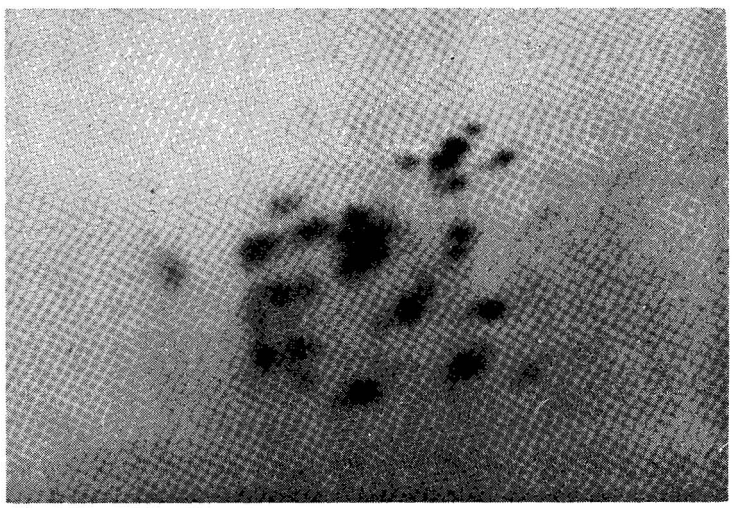

Fig. 1. Two-dimensional electrophoresis of mixed yeast tRNAs by the method of Varriccio and Ernst[7]. Start at top right. Horizontal dimension, Tris–borate–EDTA buffer (pH 8.3), 8 *M* urea. Vertical dimension, Tris–borate–EDTA buffer (pH 8.3).

It is evident that no single method can separate such a complex mixture of very similar components and that a sequential fractionation scheme will be required. The first stages of such a scheme are critical, as they should be relatively simple, capable of being scaled up to at least the gram scale, and if possible the initial fractions obtained should be suitable for introduction into the next stage without intermediate isolation of the solutes, a process which is invariably accompanied by losses and by the risk of enzymatic degradation. The main methods which have been used for tRNA separation are counter-current distribution, reversed-phase partition chromatography, ion-exchange chromatography and adsorption chromatography on materials such as hydroxyapatite and benzoyl-diethylaminoethyl (BD) cellulose. None of these satisfy all the criteria given above, and we have therefore studied in detail the method originally described by Holmes *et al.*[6]. This involves chromatography of the tRNA mixture on a column of Sepharose 4B in a gradient of decreasing $(NH_4)_2SO_4$ concentration in 10 m*M* acetate buffer (pH 4.25) containing 10 m*M* $MgCl_2$. The

method was originally applied to the fractionation of *E. coli* tRNAs, and has required modification for use with yeast tRNAs.

This paper presents an account of the optimization of the method, the subsequent use of a second stationary phase Ultrogel AcA 44, and of the separations achieved with the combined method.

2. MATERIALS AND METHODS

2.1. Materials

Sepharose grades 2B, 4B, 6B, 4B CL, Sephadex G-150 and Sephacryl S-200 were obtained from Pharmacia (London, Great Britain). Ultrogel grades AcA 34, 44 and 54 from LKB (Croydon, Great Britain). Bio-Gels P-2 and P-100 from Bio-Rad Labs. (Richmond, Calif., U.S.A.). Spheron 1000 was a gift from Dr. Z. Prusík. Phenyl-Sepharose CL was a gift from Professor S. Hjertén. "Aristar" grade $(NH_4)_2SO_4$ (BDH, Poole, Great Britain) was used for the preparation of the salt gradients.

2.2. Chromatography

Gel chromatography was carried out in jacketed glass columns of diameters 0.6–2.3 cm and length 30–50 cm. Double-ended, plunger type columns were used for the larger diameters in order to minimize dead volumes. The columns were maintained at constant temperature during packing and operation. Sepharose chromatography was carried out in 10 m*M* acetate buffer (pH 4.25–4.30) containing 10 m*M* Mg^{2+} and 0.1 % diethyl pyrocarbonate to act as a ribonuclease inhibitor. The column was packed with a suspension of the appropriate Sepharose grade in the acetate buffer and equilibrated with the same buffer containing 2.0 *M* $(NH_4)_2SO_4$ until the influent and effluent conductances were identical. The sample was dissolved in buffer and adjusted to 2.0 *M* $(NH_4)_2SO_4$ by addition of a 4.0 *M* solution of the salt in acetate buffer.

If a fraction was to be re-chromatographed directly, its electrolytic conductance was measured and solid $(NH_4)_2SO_4$ added to bring the concentration up to 2.0 *M*. The contributions of the other components of the buffer to the conductance were negligible. Sepharose columns were operated with a linear salt gradient from 2.0–0.5 *M* $(NH_4)_2SO_4$ in acetate buffer (pH 4.25) containing Mg^{2+}, while Ultrogel columns were operated in a linear gradient from 2.2–0 *M* $(NH_4)_2SO_4$ in the same buffer. Flow-rates were 11–12 ml/h·cm^2, and the total gradient volume was approximately 300 ml/cm^2 packed-bed surface area. Resolution appeared to be independent of column length above a minimum value. The tRNAs were recovered from selected fractions by exhaustive dialysis at 0° in Visking 18/32 cellulose tubing, concentration in a rotary evaporator and precipitation by the addition of two volumes of ethanol and about 4 drops of 3 *M* NaCl to the concentrate. The precipitates were stored overnight at 0°, collected by centrifugation, washed with ethanol and dried *in vacuo*.

2.3. Electrophoresis

Two-dimensional electrophoresis was carried out by the method of Varriccio and Ernst[7], in which migration in the first dimension is carried out in a $15 \times 5\%$ polyacrylamide gel containing 90 m*M* Tris–borate–EDTA buffer (pH 8.3) and 8 *M* in urea. The second migration is carried out in a $16 \times 5\%$ polyacrylamide gel slab,

using the same electrolyte without urea. Visualization of the tRNAs was achieved with the carbocyanine dye "Stains-all"[8].

One-dimensional electrophoresis was carried out on the long dimension of the LKB Multiphor gel electrophoresis apparatus, using the Tris–borate–EDTA buffer of Varriccio and Ernst[7] either with or without urea. Starts were made with 1×0.15 cm pieces of Schleicher & Schüll 2043a paper, wetted with solutions containing 0.5–2.0 μg tRNA, and dipped in liquid $16 \times 5\%$ polyacrylamide gel mixture immediately before laying on the starting position at the cathodic end of the gel slab. This starting method gave extremely good resolution of adjacent zones. Electrophoresis was carried out at constant power of 25 W (usually 750 V, 30–40 mA) at a coolant temperature of 10°.

2.4. Assay of amino acid acceptance activity

Assays were carried out as described by Hoskinson and Khorana[9] with minor changes as described by Gillam *et al.*[10] and by Holness and Atfield[11], using their cacodylate–ATP–Mg^{2+} buffer system, and incubating for 25 min at 38°. An aliquot was transferred to a paper disc, washed with 5% aqueous trichloroacetic acid, ethanol and ether, and radioactivity determined in a scintillation spectrometer, using a diphenylcarbazole–toluene scintillation fluid.

3. RESULTS

The effects of a number of changes in operating conditions were investigated in detail. The influence of pH was very marked. Insolubility in strong salt solutions precludes operation below about 3.3, as the isoelectric points of the tRNAs lie in the range 2.6–2.9 (ref. 12). The tRNAs are insufficiently retarded to give adequate resolution above pH 5. The operating pH is, however, critical for resolution in the latter part of the chromatographic profile (compare Fig. 6). Zones D and E were well resolved at pH 4.25, poorly resolved at pH 4.4, and merged into a single zone at pH 4.6. At pH 3.95, zone E although resolved from zone D trailed badly. The effect of pH in the Ultrogel system differed from that in the Sepharose system. In the former case changes of profile in the pH 4.0–4.6 range were more evident in the B and C region, changes in the latter part of the profile being less marked. The influence of pH on Ultrogel chromatography is obviously complex and requires further study.

The choice of chromatographic stationary phase was also very important. Fig. 2 shows that Sepharose 4B gave the best resolution under the standardized operating conditions, Sepharose 6B being only slightly worse, while Sepharose 2B and the cross-linked agarose gel Sepharose 4B CL were very inferior. Sephadex G-150, Sephacryl S-200 and the two beaded polyacrylamide gels Bio-Gel P-2 and P-100 gave virtually no fractionation. Phenyl-Sepharose 4B CL and Spheron 1000 gave some resolution but were inferior to Sepharose 4B. The composite agarose–polyacrylamide gel Ultrogel was however useful as shown in Fig. 3. In the manufacturers' AcA nomenclature the first numeral denotes the polyacrylamide content, while the second numeral indicates the agarose content. We examined only the 4% agarose grades AcA 34, 44 and 54. AcA 34 gave poor resolution with low retention. The less porous Ultrogel grades AcA 44 and 54 gave useful separations, similar to but distinct from those obtained with Sepharose 4B. Ultrogel AcA 44 proved to be very valuable for the further resolution of Sepharose 4B fractions.

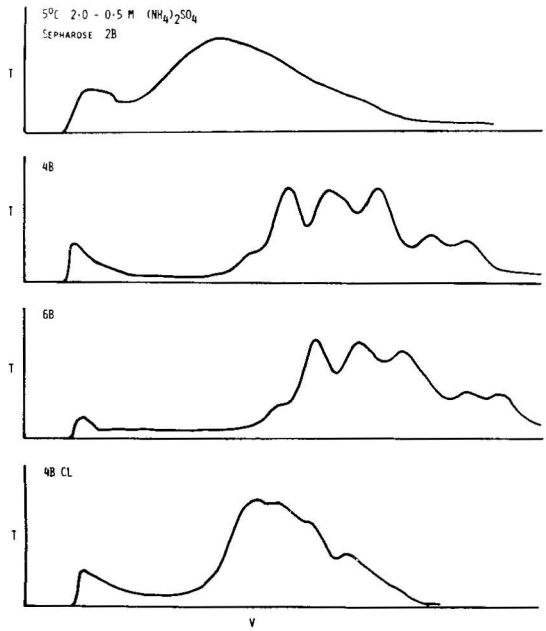

Fig. 2. Separation of mixed yeast tRNAs in a $(NH_4)_2SO_4$ gradient, 2.0–0.5 *M* on Sepharose 2B, 4B, 6B and 4B CL.

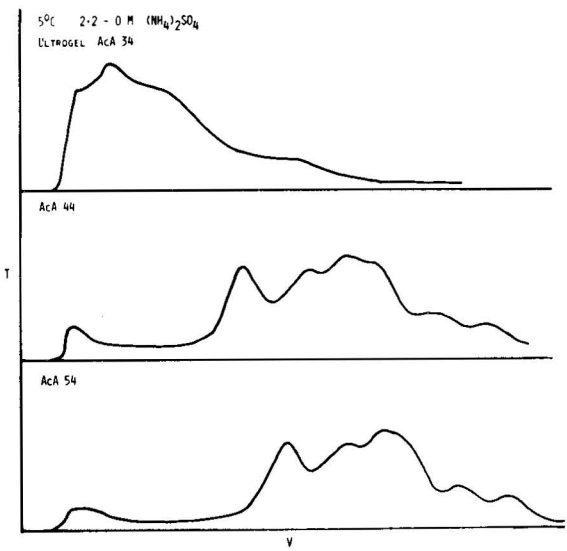

Fig. 3. Separation of mixed yeast tRNAs in a $(NH_4)_2SO_4$ gradient, 2.2–0 *M*, 5°, on Ultrogels AcA 34, 44 and 54.

The operating temperature also had a marked influence on these separations as shown in Fig. 4 for Sepharose 4B under the standard conditions at 5°, 10°, 20° and 35°. It is evident that both the retention volumes of individual zones and the distance between them decreases with increasing temperature, although the latter

effect is not uniform in the profile. These results are extremely important, since they indicate that the retention process is exothermic, and thus the free energy change of binding is primarily enthalpic rather than entropic. This in turn suggests that the binding mechanism is probably adsorption rather than hydrophobic interaction, a conclusion confirmed by the absence of hydrophobic sites in agarose. It will be shown later that this differential temperature effect may be used for fractionation.

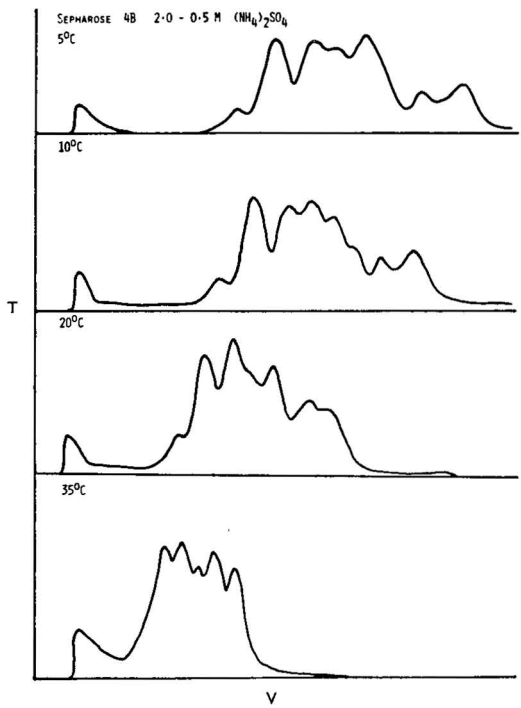

Fig. 4. Separation of mixed yeast tRNAs in a $(NH_4)_2SO_4$ gradient, 2.0–0.5 *M*, on Sepharose 4B at 5°, 10°, 20° and 35°.

Fig. 5 shows the similar temperature effect on retention by Ultrogel AcA 44, which in spite of the hydrophobic sites provided by the polyacrylamide content, also shows a negative temperature coefficient of binding. The even more hydrophobic phenyl-Sepharose 4B CL also has a negative temperature coefficient.

A typical separation of yeast tRNAs on Sepharose 4B under the standardized conditions of pH 4.25, 5°, and a flow-rate of 11–12 ml/h·cm² is shown in Fig. 6. This chromatographic profile was very reproducible over a wide range of column sizes and solute loads. Seven major zones are evident. The first zone PP, which is only slightly retarded, contains degraded RNA and will not be considered further here.

Fraction PreA was re-chromatographed twice on Sepharose 4B by the $(NH_4)_2SO_4$ concentration adjustment technique to reduce contamination with fraction A. The final chromatogram is shown in Fig. 7. This fraction contains high methionine acceptor activity, with minor contamination with arginine and valine acceptor activities. Gel electrophoresis (Fig. 8) confirms that this is a highly purified

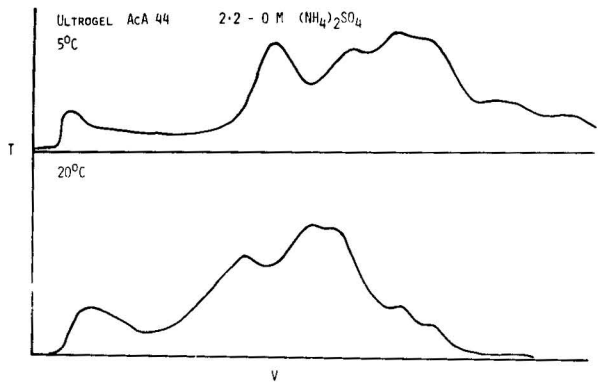

Fig. 5. Separation of mixed yeast tRNAs in a $(NH_4)_2SO_4$ gradient, 2.2–0 *M*, on Ultrogel AcA 44 at 5° and 20°.

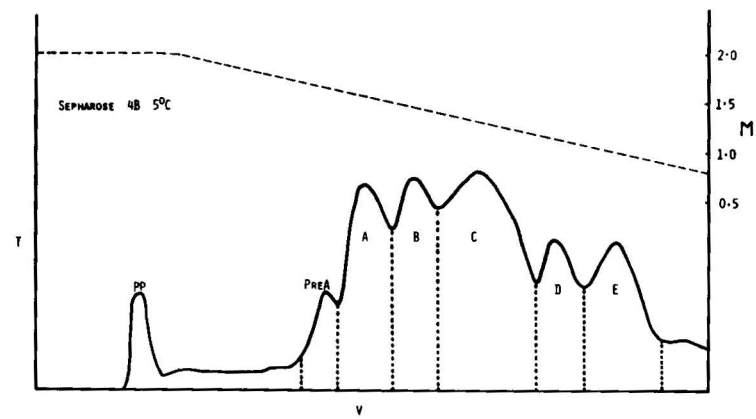

Fig. 6. Preparative separation of mixed yeast tRNAs on Sepharose 4B at 5° in a 2.0–0.5 *M* $(NH_4)_2SO_4$ gradient (— — —).

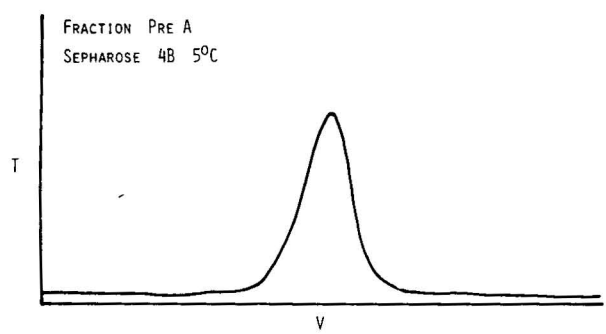

Fig. 7. Second re-chromatography of Fraction PreA on Sepharose 4B at 5° in a 2.0–0.5 *M* $(NH_4)_2SO_4$ gradient.

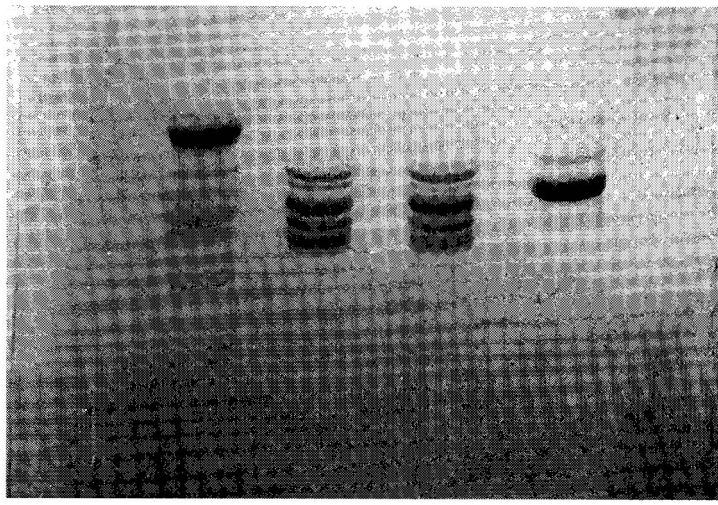

Fig. 8. Electrophoresis of Fractions PreA, A1 (front), A1 (rear) and A2 (left to right) at pH 8.3, 8 *M* urea.

$tRNA^{Met}$, which should readily be purified to homogeneity on an alternative chromatographic medium such as BD-cellulose[10].

Fraction A was re-run on Sepharose 4B to reduce contamination with fraction B, and then chromatographed on Ultrogel AcA 44 at 5° and in a 2.2–0 *M* $(NH_4)_2SO_4$ gradient to give two fractions A1 and A2 (Fig. 9). Fraction A1 had high tryptophan acceptor activity and substantial acceptor activities for threonine, arginine and valine. Fraction A2 had very high asparagine acceptor activity, with minor contamination with threonine, valine and lysine acceptor activity. This is confirmed by the gel electrophoresis of this fraction (Fig. 8). Samples from the front and rear of zone A1 together with a sample of zone A2 were run in this gel. Differences in acceptor activity and electrophoretic pattern through zone A1 appeared to be small. Contamination of the $tRNA^{Asn}$ in zone A2 was possibly due to traces of A1, and could probably be removed by re-chromatography on Ultrogel or in an alternative system.

Fraction B was extremely complex both in acceptor activities and electrophoretic pattern. It was enriched in glutamine, isoleucine and glycine acceptor activities but was refractory to further fractionation on either polysaccharide gel system.

Fraction C was also fairly complex, and could not be fractionated further on Ultrogel columns. It could however be separated into three fractions by making use of the temperature effect on Sepharose 4B. A fraction C obtained by an initial chromatography at 5° on Sepharose 4B was re-run after $(NH_4)_2SO_4$ concentration adjustment at 20° on the same stationary phase, with the results shown in Fig. 10. Fraction C1 was highly enriched in cysteine acceptor activity, which could be purified to homogeneity by chromatography on a reversed-phase partition system as shown by Holness and Atfield[11]. Fraction C2A was mainly enriched in tyrosine acceptor activity, but also contained substantial amounts of arginine, leucine and histidine acceptor activities. Fraction C2B also contained histidine and leucine acceptor activities. The electrophoretic patterns of the C fractions were clearly complex (Fig. 11).

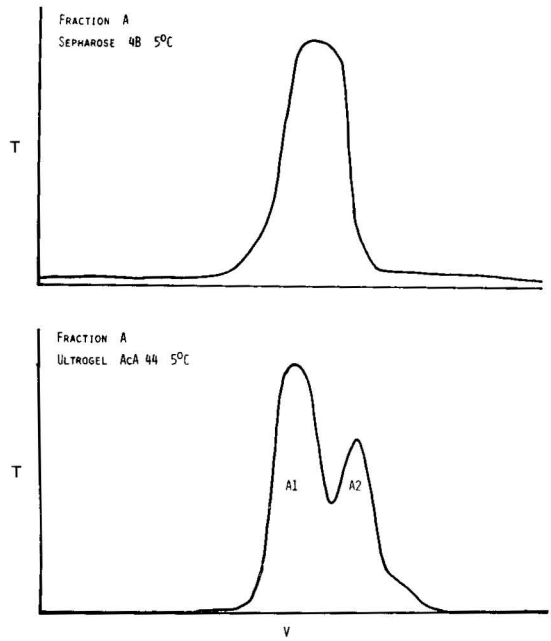

Fig. 9. Top: re-chromatography of Fraction A on Sepharose 4B at 5°, 2.0–0.5 *M* $(NH_4)_2SO_4$ gradient. Bottom: re-chromatography of purified Fraction A on Ultrogel AcA 44 at 5°, 2.2–0 *M* $(NH_4)_2SO_4$ gradient.

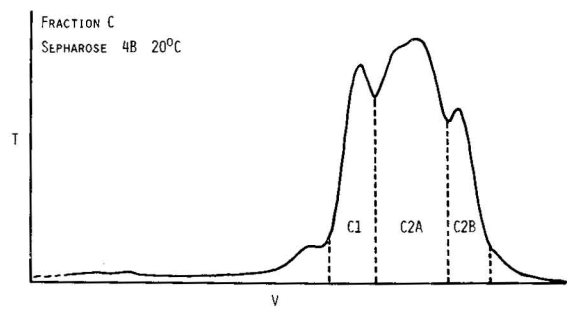

Fig. 10. Re-chromatography of fraction C on Sepharose 4B at 20°, in a 2.0–0.5 *M* $(NH_4)_2SO_4$ gradient.

Fraction D was less complex, and could be further separated on Ultrogel AcA 44 at 5° into two fractions D1 and D2 as shown in Fig. 12. Fraction D1 contains the major part of the histidine acceptor activity of the total tRNA mixture, but several minor contaminants including proline and lysine acceptor activities. Fraction D2 also contains high lysine and histidine activities. The electrophoresis diagram confirms that the last separation is relatively inefficient (Fig. 13).

Fraction E was relatively simple in composition and was re-run on Sepharose 4B at 5° to reduce contamination with Fraction D. It was further fractionated on Ultrogel AcA 44 at 5° in a 2.2–0 *M* $(NH_4)_2SO_4$ gradient to give three fractions as

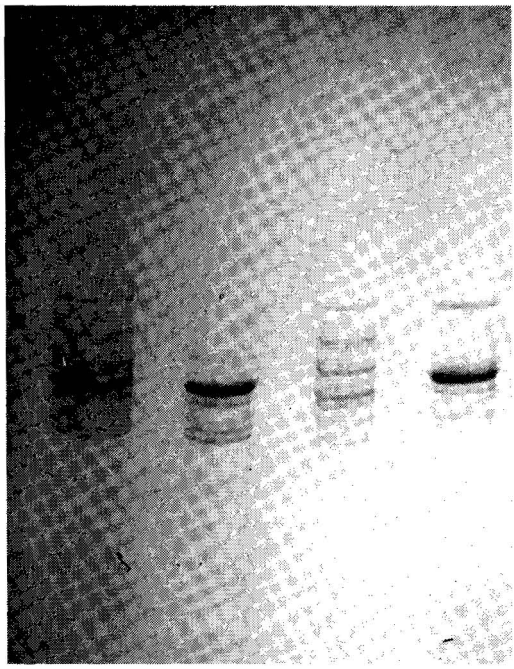

Fig. 11. Electrophoresis of (left to right) fractions C, C1, C2A and C2B at pH 8.3, 8 *M* urea.

shown in Fig. 14. Fractions E1 and E3 were relatively pure as demonstrated by the electrophoretic patterns shown in Fig. 15. Fraction E1 contains mainly proline acceptor activity with minor histidine acceptor activity. It should be relatively easy to purify to homogeneity. E2 is complex with high serine and proline acceptor activity, and minor alanine acceptor activity. The serine activity is probably associated with the three slow moving bands shown in Fig. 15. Fraction E3 appears to be virtually homogeneous by electrophoresis and amino acid acceptor assay. It is a $tRNA^{Ala}$ species, and could probably be purified from trace impurities by chromatography on BD cellulose. The results of these separations are summarised in Table 1.

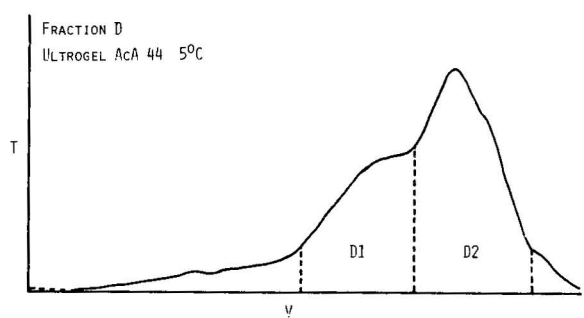

Fig. 12. Chromatography of fraction D on Ultrogel AcA 44 at 5°, in a 2.2–0.0 *M* $(NH_4)_2SO_4$ gradient.

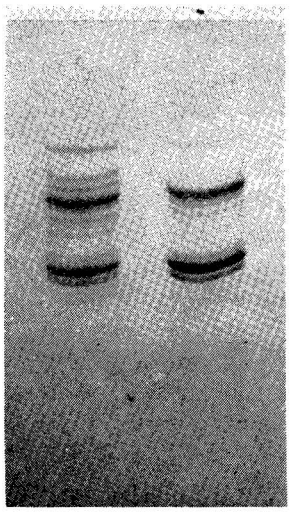

Fig. 13. Electrophoresis of (left to right) fractions D1 and D2 at pH 8.3, 8 *M* urea.

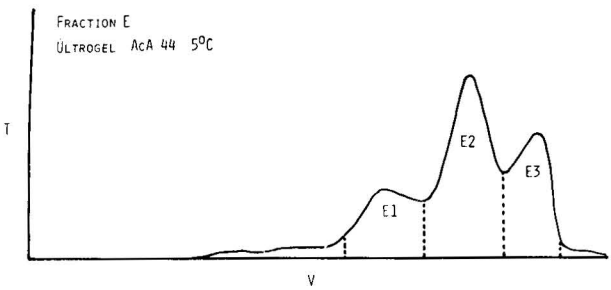

Fig. 14. Re-chromatography of purified Fraction E on Ultrogel AcA 44 at 5° in a 2.2–0 *M* $(NH_4)_2SO_4$ gradient.

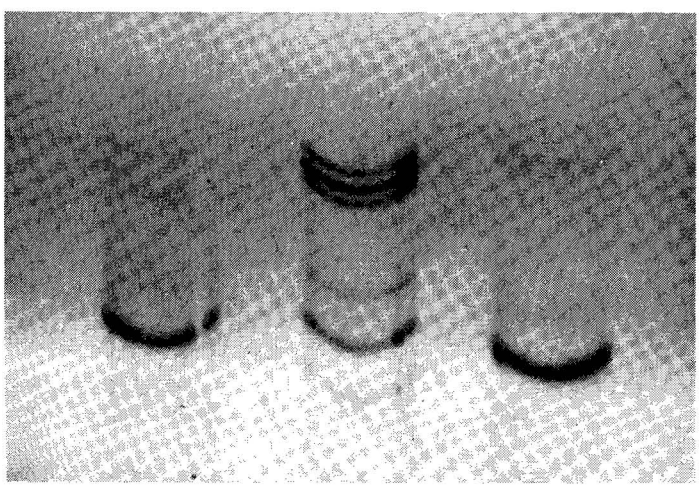

Fig. 15. Electrophoresis of (left to right) fractions E1, E2 and E3 at pH 8.3, no urea.

TABLE 1

AMINO ACID ACCEPTANCE OF SEPHAROSE AND ULTROGEL FRACTIONS

Glutamic and aspartic acid acceptor assays were unsatisfactory, and the results are not included in the Table. Results are graded from + (significant) to ++++ (very high acceptor activity).

Amino acid	*Fraction*											
	PreA	*A1*	*A2*	*B*	*C1*	*C2A*	*C2B*	*D1*	*D2*	*E1*	*E2*	*E3*
Alanine					+			+	+		+	++++
Arginine	+	+++				+-						
Asparagine		+-	+-+-									
Cysteine					+++-	-						
Glutamine				+++-	+	-+						
Glycine				++--	+	-+	--					
Histidine						+-	+-	+-+	-+	--		
Isoleucine				++-		-	--					
Leucine					-	--+	--					
Lysine			+		-			+	--			
Methionine	+--+-	+		+				+				
Phenylalanine						-						
Proline								-	--	-+++	++	
Serine						+--	+-		-+		-++	
Threonine		-++	--									
Tryptophane		+++-										
Tyrosine						+-+	--					
Valine	+	++	+			+		+				

4. DISCUSSION

The relatively simple chromatographic system described here can provide relatively pure preparations of tRNAs for four acceptor activities, *viz.*, for alanine, asparagine, methionine and proline, and enriched concentrates for two further species, tryptophane and cysteine.

Its particular advantages for preparative purposes lies both in the ability to scale up with reproducibility of the chromatographic profile, and the almost unique facility with which chosen fractions can be re-chromatographed without desalting or isolation of the solutes, by simple adjustment of their salt concentrations to the chosen initial value (2.0 or 2.2 *M*). Ultrogel AcA 44 provides a useful alternative to Sepharose 4B for this purpose. This facility for re-chromatography makes it profitable to explore multiple re-runs on a single stationary phase as a means of eliminating minor impurities. This method has not been fully explored in the past, since the necessity for desalting and recovery of solutes between stages makes it more attractive to investigate alternative stationary phases. It must however be emphasized that the advantages of cascade operation apply equally to multiple re-chromatography on a single phase.

The mildness of the operating conditions is demonstrated by the fact that both Holmes *et al.*[6] and Nygard and Hulten[13] have been able to chromatograph aminoacyl tRNAs in the Sepharose 4B system with good recoveries.

The mechanism of tRNA chromatography on these neutral polysaccharide columns remains obscure. Molecular sieving effects appear to be unimportant, since although Sepharose 4B (exclusion limit $\approx 3 \cdot 10^6$) gives optimal separations, Ultrogel AcA 54 (exclusion limit $\approx 7 \cdot 10^4$) also gives good resolution. Sepharose 2B (exclusion limit $\approx 25 \cdot 10^6$) gives very poor resolution. Cross-linked gels appear to give poor separations both in the polygalactose series (Sepharose 4B CL) and in the polyglucose series (Sephacryl S 200). Satisfactory chromatography has in fact only been demonstrated with agarose derivatives with two free *cis* hydroxyl groups in a galactose unit. Since the exothermic nature of the retention process on both stationary phases has been demonstrated, either an adsorptive or a selective solubility retention mechanism appears to be indicated. The marked increase of the binding of the E fraction to Sepharose 4B over the narrow pH range 4.0–4.4 favours the latter mechanism as the rapidly increasing ionisation of the cytosine (pK_a 4.1) and adenosine (pK_a 3.8) amino groups in this pH range will tend to reduce the net charge on the tRNA molecule, and thus to reduce its solubility in strong salt solutions. Mixed tRNAs are in fact virtually insoluble in 2.0 *M* $(NH_4)_2SO_4$ in their isoelectric region of pH 2.6–2.9, even in the absence of gel media. However, since it has been demonstrated in this work that successful chromatography of the tRNAs is critically dependent on the nature of the gel stationary phase employed, it is very probable that both the extent and the steric configuration of the accessible internal surfaces within the gel bead are extremely important in the retention process. Unfortunately these factors are very difficult to assess independently.

5. ACKNOWLEDGEMENTS

I am indebted to my wife, Mrs. P. Morris, for the greater part of the experimental work on which this paper is based, and to K. W. M. Davy for carrying out the amino acid acceptance assays.

6. SUMMARY

A sequential scheme for the fractionation of yeast transfer RNAs by chromatography on the polysaccharide gel media Sepharose 4B and Ultrogel AcA 44 in reversed concentration gradients of ammonium sulphate is described and the optimum conditions worked out. Four species, $tRNA^{Met}$, $tRNA^{Asn}$, $tRNA^{Pro}$ and $tRNA^{Ala}$ are obtained in a highly purified form, while several other acceptor species are highly concentrated in the subfractions. The mechanism of this chromatographic system is discussed.

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FRACTIONATION OF PROTEINS ON SEPHAROSE AT LOW pH AND ON POLYTETRAFLUOROETHYLENE

STELLAN HJERTÉN

Institute of Biochemistry, University of Uppsala, Biomedical Centre, Box 576, S-751 23 Uppsala (Sweden)

CONTENTS

1. INTRODUCTION

Many of the observations and findings presented here on the fractionation of proteins at low pH on columns of Sepharose and polytetrafluoroethylene (PTFE) are several years old, but they have not been presented previously because we have no satisfactory explanation of the mechanism behind the separations obtained, except for the case of Sepharose. Therefore, the author would welcome comments regarding the nature of the interaction between the adsorbents and the proteins.

2. SEPHAROSE EQUILIBRATED IN BUTANOL–ACETIC ACID–WATER

Around 1970, we began our studies on the structure of biological cell membranes[1]. To solubilize the membranes efficiently we used detergents, which interacted strongly with the membrane proteins and brought them into solution (these studies gave us the idea of hydrophobic interaction chromatography, a method which is the subject of several papers presented at this symposium). However, there are some drawbacks to the use of detergents so we also investigated the use of organic solvents for solubilization of the membranes.

We found that a heavily opalescent suspension of erythrocyte membranes became completely transparent upon transfer to a mixture of equal volumes of butanol, acetic acid and water. When this membrane solution was applied to a column of Sepharose 4B equilibrated with the same butanol–acetic acid–water mixture, most of the membrane material was strongly adsorbed and could not be desorbed by a stepwise increase in ionic strength. Similar results were obtained when the acetic acid in the above experiment was replaced with morpholine. The pH of the butanol–acetic acid–water (1:1:1) mixture was 2.2, as measured with a glass electrode, and

that of butanol–morpholine–water (1:1:1) was 11.0. Accordingly, in an aqueous butanol solution there is a strong adsorption of membrane proteins to Sepharose at both low and high pH.

In order to utilize this interaction for the development of a new fractionation method, one must learn more about the separation mechanism. The first obvious question is whether the separations obtained are due to an interaction of an electrostatic nature. To test this we prepared agarose with a very low content of sulphate and carboxylic groups using method 3b in ref. 2 combined with a desulphation step[3,4]. The adsorption was then less, but still strong. This finding seems to indicate that electrostatic interactions contribute to the adsorption, but that additional forces are involved. When we added sodium acetate at a concentration of 0.06 *M* to the butanol–acetic acid–water mixture to suppress the electrostatic interaction (we could not use sodium acetate in a higher concentration than 0.06 *M* as the membrane proteins then started to precipitate) the adsorption was still considerable. This finding supports the view that the main interaction is not electrostatic in nature.

In fact, it is difficult to find a medium that desorbs the membrane proteins completely. The main problem is associated with the requirement that the medium must not precipitate the proteins. After many trial-and-error experiments we found that replacement of butanol with propanol greatly reduced the adsorption (Fig. 1). A glycine–sodium hydroxide buffer, pH 9.8, containing sodium dodecyl sulphate (SDS) appeared to displace at least part of the membrane proteins. There accordingly exist media that permit desorption of the proteins, a fact which opens up the possibility of developing a new separation method. Fractionation in the butanol–acetic acid–

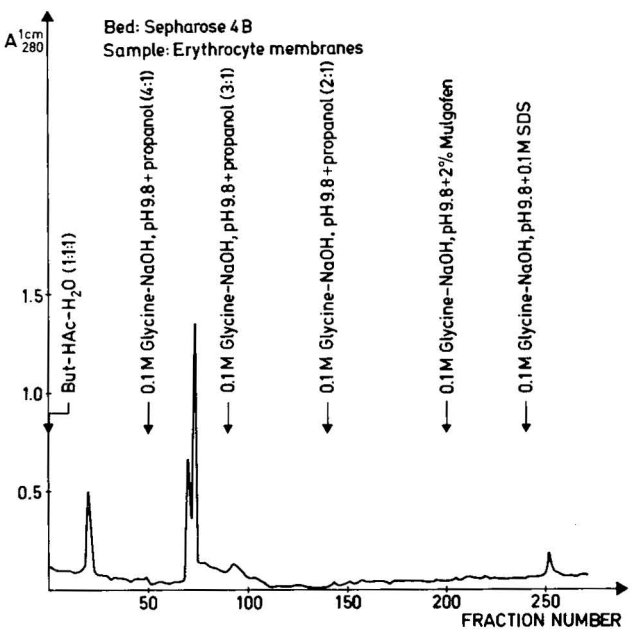


Fig. 1. Chromatographic behaviour of human erythrocytes (17 mg) on a column (28 × 1.4 cm) of Sepharose 4B, equilibrated with butanol–acetic acid–water (1:1:1). A similar experiment with an analysis of the chromatogram is given elsewhere[5].

water system is not discussed further here, but it is considered in the paper on the fractionation of membrane proteins[5].

3. SEPHAROSE EQUILIBRATED IN 0.01 *M* SODIUM ACETATE, pH 4.0

Water-soluble proteins, such as plasma proteins, are not soluble in butanol–acetic acid (morpholine)–water and could not be utilized as model proteins to study the nature of the adsorption of proteins to Sepharose in these media. However, proteins do adsorb to Sepharose at low pH even in the absence of butanol. This is exemplified in Fig. 2, which shows a fractionation of human plasma on Sepharose 4B. The column was equilibrated with 0.01 *M* sodium acetate, pH 4.0. The proteins were desorbed by stepwise increases in pH. When the experiment was performed in 0.06 *M* sodium acetate, pH 4.0, instead of 0.01 *M* sodium acetate, pH 4.0, no proteins were adsorbed. Nor was there any adsorption in 0.01 *M* sodium acetate, pH 4.0, when Sepharose was replaced with an agarose of extremely low sulphate and carboxyl content.

These observations indicate that the charged groups on the agarose are of importance for the adsorption. Agarose contains both sulphate and carboxyl groups. To determine whether either (or both) of these groups in Sepharose might be responsible for the adsorption of proteins at low pH, we studied the behaviour of human plasma on two ion exchangers, one with sulphonate [sulphoethyl(SE)-Sephadex] and one with carboxyl [carboxymethyl(CM)-Sephadex] groups.

From the chromatograms shown in Fig. 3, it is evident that plasma proteins are strongly adsorbed at pH 4 to SE-Sephadex but only slightly to CM-Sephadex. From these experiments one might conclude that the sulphate rather than the carboxyl groups of the Sepharose are mainly responsible for the adsorption. However, the adsorption of plasma proteins to CM-Sepharose (Fig. 4) is considerably stronger than to Sepharose (Fig. 2). Probably both the sulphate and the carboxyl groups therefore contribute to the adsorption to Sepharose at low pH.

The question then arises as to whether the interaction can be mainly ascribed to a true coulombic interaction or to other interactions such as hydrogen bonding. To test this we analysed by agarose gel electrophoresis (Fig. 5) the material corresponding to the different peaks obtained on Sepharose 4B. As shown, the serum proteins are obviously eluted in the order of their electrophoretic mobilities or, in other words, the order of their surface charge densities. The same result was obtained on CM-Sepharose. These findings indicate that the mechanism of adsorption of proteins to Sepharose at low pH is the same as in ion-exchange chromatography, *i.e.*, the interaction is of an electrostatic nature. Our investigations therefore seem to show that one cannot introduce a new separation parameter by performing a chromatographic experiment on Sepharose at low pH, as we had hoped. However, these negative results can also be taken advantage of in the following two ways:

(1) A very inexpensive ion exchanger could be produced based on crude agar. The result of such an experiment on a column prepared from a commercial agar is shown in Fig. 6 (the gel grains were obtained by pressing a 4% gel through a net as described in ref. 6).

(2) The fact that Sepharose acts as an ion exchanger at low pH opens up the possibility of combining ion-exchange chromatography and hydrophobic interaction

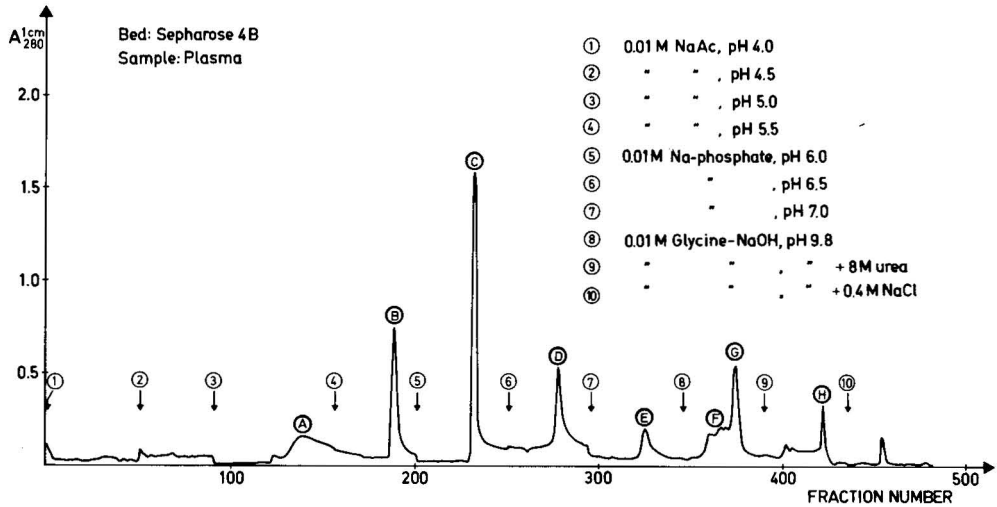


Fig. 2. Chromatographic behaviour of normal human plasma (1 ml) on a column (38 × 1.4 cm) of Sepharose 4B equilibrated with 0.01 *M* sodium acetate, pH 4.0. (The buffers given in this and other figures are those used for desorption. This and similar experiments were run at 20° and a flow-rate of 6 ml/h.)

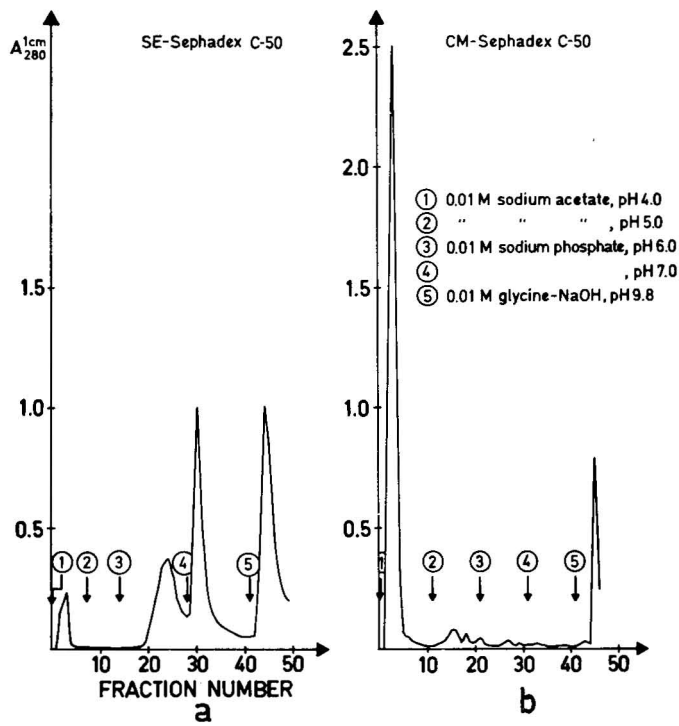


Fig. 3. Chromatographic behaviour of human plasma (0.2 ml) on a column (10 × 0.8 cm) of SE-Sephadex (a) and CM-Sephadex (b), run under identical experimental conditions. The capacities of the ion exchangers were 2.1 and 4.7 mequiv./g, respectively.

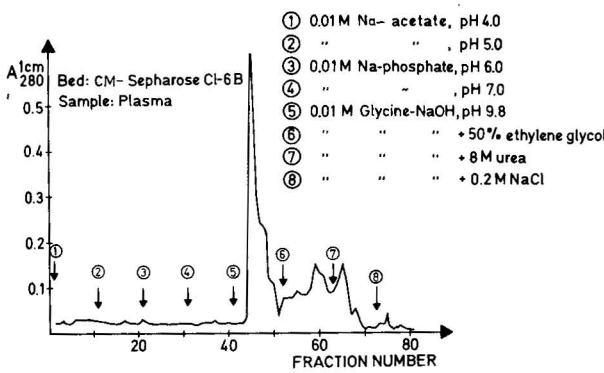


Fig. 4. Behaviour of human plasma (10.2 ml) on a column (10 × 0.8 cm) of CM-Sepharose CL-6B. The capacity of the ion exchanger was 0.12 mequiv./ml gel bed. The adsorption is much stronger than on CM-Sephadex (Fig. 3b) and Sepharose (Fig. 2).

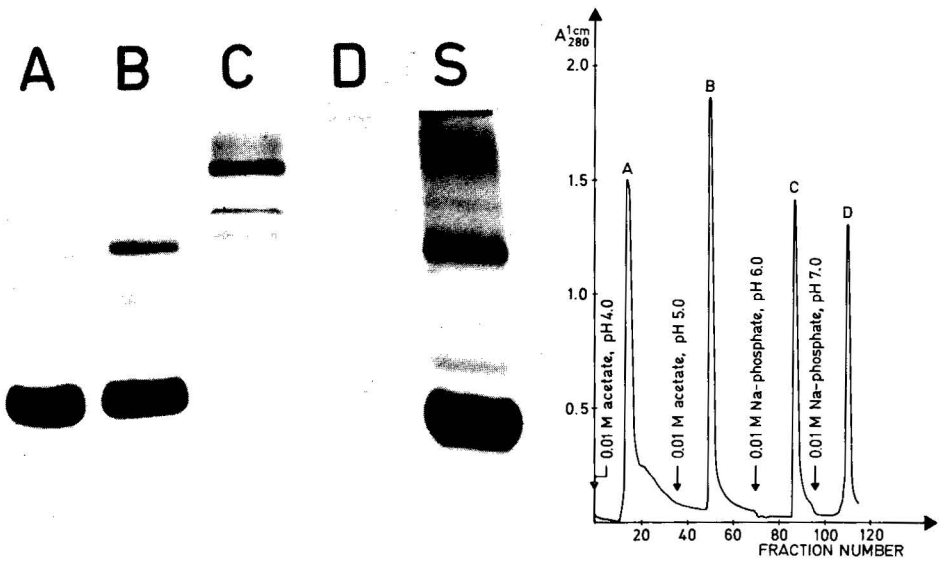
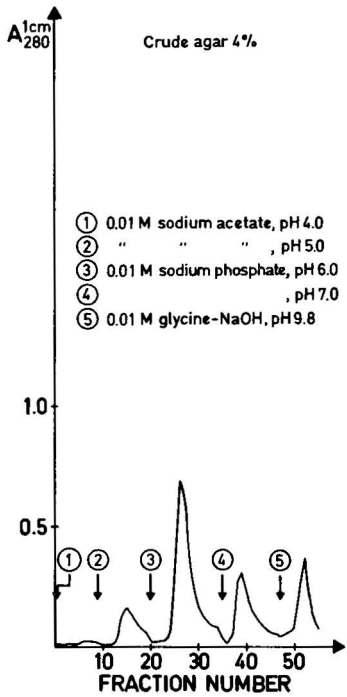


Fig. 5. Analysis by agarose gel electrophoresis of the fractions obtained when human plasma is fractionated on Sepharose 4B equilibrated with 0.01 *M* sodium acetate, pH 4.0 (the chromatogram is on the right). The proteins are eluted in order of decreasing mobilities, *i.e.*, the same order as is obtained in ion-exchange chromatography.

chromatography by running amphiphilic agarose columns at low pH. An example is shown in Fig. 7.

As mentioned, Sepharose behaves as an ion exchanger at low pH. Hydrogen bonds are probably not involved to a great extent. This was supported by the observation that proteins adsorbed to Sepharose in 0.01 *M* acetate, pH 4, were not desorbed when the buffer was supplemented with urea (8 *M*) or ethylene glycol (50%). The hydroxyl groups in the galactose units of the agarose appear not to be good proton donors for hydrogen bond formation. What groups other than hydroxyl groups might



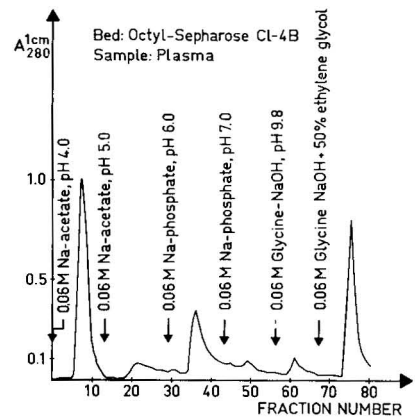


Fig. 6. Behaviour of human plasma (0.2 ml) on a column (10 × 0.8 cm) prepared from commercial agar and equilibrated at pH 4.0. The results is similar to that obtained on SE-Sephadex (Fig. 3a). The capacity of the column was about 0.3 mmole of sulphate per gram of agar (= 0.012 mmole of sulphate per ml gel bed).

Fig. 7. A combination of ion-exchange chromatography and hydrophobic interaction chromatography by performing an experiment on octyl-Sepharose, equilibrated at a low pH.

be suitable for hydrogen-bond chromatography? We have investigated whether allantoin (a urea derivative) coupled to Sepharose could be utilized for such purposes. The chromatogram obtained with plasma on this material was similar to that shown in Fig. 2. Agarose gel electrophoresis showed that the order of elution of the plasma proteins was the same as for Sepharose 4B (Fig. 5). The allantoin groups in the Sepharose derivative thus had no effect on the fractionation, *i.e.*, they did not act as sites for adsorption.

4. POLYTETRAFLUOROETHYLENE EQUILIBRATED AT pH 4 AND HIGHER

We have also investigated the possibility of adsorbing proteins to PTFE at low pH. This plastic powder was obtained from Imperial Chemical Industries, Great Britain, under the Trade-name Fluon, L 169 A.

A column (35 × 1.4 cm), packed with PTFE, was equilibrated with 0.01 *M* sodium acetate, pH 4.0, and 2 ml of human plasma, dialyzed against this buffer, were applied. The adsorbed proteins could not be desorbed by increasing the pH or the ionic strength or by adding urea to the buffer; only SDS displaced the proteins from the column (Fig. 8). The proteins are accordingly very strongly adsorbed to PTFE if

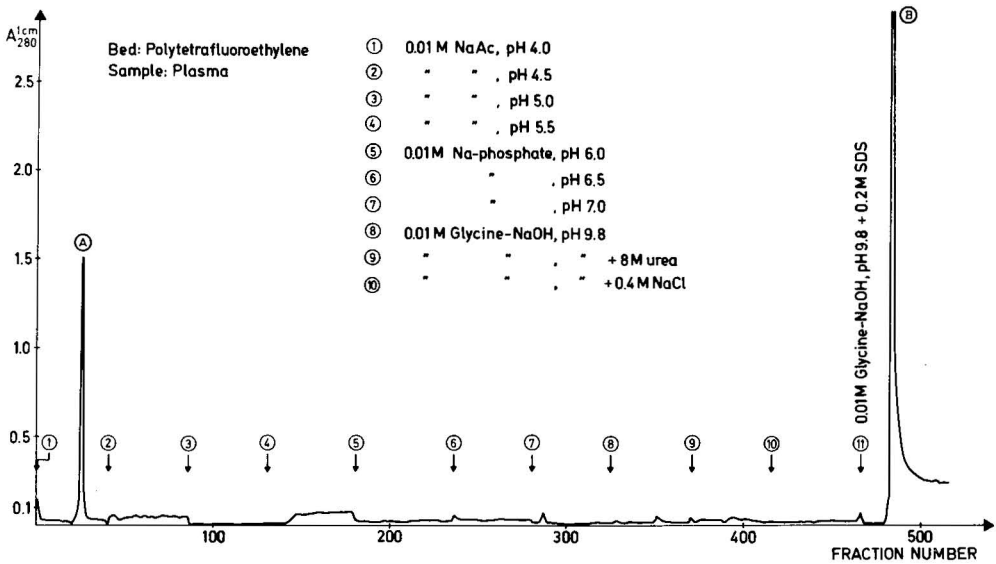


Fig. 8. Chromatogram showing the very strong adsorption of plasma proteins to a column of PTFE equilibrated at pH 4.0. When the column was equilibrated at pH 7.5 only very slight adsorption could be detected, suggesting that some pH between 4 and 7.5 might give an adsorption appropriate for protein fractionation.

the column is equilibrated at pH 4. However, if the column is equilibrated at pH 7.5 (0.01 *M* sodium phosphate) the adsorption is weak. It should therefore be possible to find a pH between 4 and 7.5 that gives a moderate adsorption useful for separation purposes. Such experiments are planned. However, it should be interesting to know the basis for the strong affinity to polytetrafluoroethylene at pH 4. The interaction is probably not electrostatic as the bed should not contain any charged groups. This is supported by the fact that desorption was not favoured by an increase in pH or ionic strength, as Fig. 8 indicates. Another possible adsorption mechanism is hydrophobic interaction, but there are some observations which go against this hypothesis.

(1) Plasma proteins interact very strongly with amphiphilic agarose derivatives (for instance, pentyl- or octyl-Sepharose) when the column has been equilibrated with a buffer of neutral pH containing salts, for instance 4 *M* sodium chloride[7]. Under these conditions only a few plasma proteins are adsorbed to PTFE.

(2) Transfer RNA is not, or is only very weakly, adsorbed to octyl-Sepharose equilibrated with 0.002 *M* sodium phosphate, pH 6.8, containing 2 *M* sodium chloride but is very strongly adsorbed to PTFE (Fig. 9).

(3) Albumin and β-lipoprotein emerge first, before γ-globulin, from a column on PTFE. On pentyl-Sepharose the elution order is reversed[7].

(4) Neither proteins (Fig. 8) nor t-RNA (Fig. 9) could be desorbed by alterations in ionic strength or pH, indicating that hydrophobic or electrostatic interactions are not responsible for the adsorption. Could both of these interactions act simultaneously on the protein molecules? This is theoretically possible, because although the electrostatic interaction is decreased upon increasing the ionic strength,

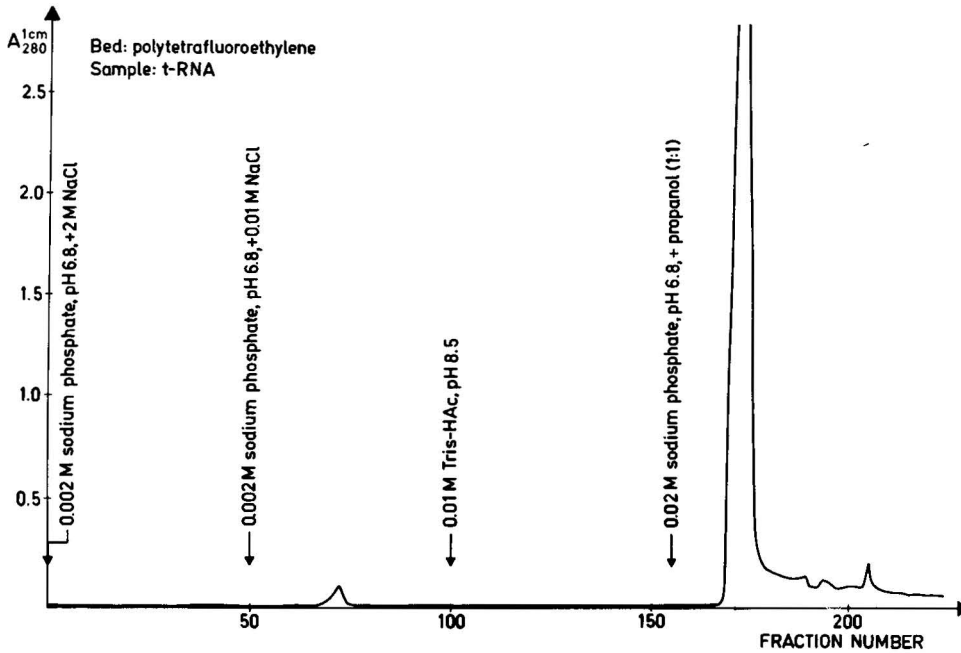


Fig. 9. Chromatogram showing the very strong adsorption of t-RNA to a column of PTFE. Desorption is effected by the addition of propanol, a finding which is being utilized to desalt t-RNA[8].

the hydrophobic interaction increases, which of course may hinder desorption. This seems unlikely, however, because *none* of the plasma proteins could be desorbed by changing the pH and the ionic strength of the eluting medium.

Could hydrogen bonds be responsible for the strong adsorption to PTFE at pH 4? Fluorine is strongly electronegative, and easily forms hydrogen bonds, but probably not in a polymer of this type. Although the nature of the adsorption to PTFE is still obscure, we have had sufficient experience with proteins and nucleic acids on this adsorbent to justify fractionation studies. The above experiments already show that PTFE can be used to desalt t-RNA (suggested by Prof. C. J. O. R. Morris at this symposium[8]).

5. COMMENTS

Although the fractionation of proteins on Sepharose in 0.01 *M* acetate at pH 4.0 appears to be based upon ion exchange, it should be pointed out that the matrix itself often has a pronounced influence on the adsorption. For instance, we have found that plasma proteins show very little adsorption to CM-Sephadex at pH 4 (Fig. 3b), but adsorb strongly to CM-cellulose.

A Sepharose column equilibrated with 0.06 *M* sodium acetate, pH 4.0, shows very little adsorption of plasma proteins, but adsorbs them very strongly when equilibrated with 0.01 *M* sodium acetate, pH 4.0. One might therefore expect that plasma proteins adsorbed to Sepharose equilibrated in the latter buffer should be desorbed by increasing the molarity of the acetate buffer to 0.06 *M*. However, this does not occur, and a considerably higher buffer concentration is required. Similarly,

proteins adsorbed to PTFE at pH 4.0 cannot be desorbed by increasing the pH to 7.5 although very few plasma proteins are adsorbed to a PTFE column equilibrated with a buffer of pH 7.5. Similar observations have been made for hydroxyapatite and amphiphilic derivatives of Sepharose used for hydrophobic interaction chromatography. The phenomenon might be a general one in ion-exchange and adsorption chromatography when polymers are adsorbed and can be expected when we are dealing with multi-point interactions.

6. ACKNOWLEDGEMENTS

The allantoin derivative of Sepharose was synthesized by Dr. Jan Rosengren, Pharmacia Fine Chemicals, Uppsala, Sweden. The author is much indebted to Mrs. Karin Elenbring for skilful technical assistance. The work was supported by the Swedish Natural Science Research Council.

7. SUMMARY

Plasma proteins can be fractionated on columns of Sepharose equilibrated with 0.01 *M* sodium acetate, pH 4.0. The adsorption is probably due mainly to the presence of sulphate and carboxylic groups in the matrix. The use of polytetrafluoroethylene as an adsorbent for the purification of proteins and t-RNA is being explored.

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NON-IONIC ADSORPTION CHROMATOGRAPHY OF PROTEINS

B. H. J. HOFSTEE and N. FRANK OTILLIO
Biochemistry Division, Palo Alto Medical Research Foundation, Palo Alto, Calif. 94301 (U.S.A.)

CONTENTS

1. INTRODUCTION

Until relatively recently it was assumed that nearly all hydrophobic amino acid side-chains of a protein are located in the interior of the molecule. The occurrence of accessible hydrophobic groups was looked upon as rather unusual. However, several years ago, Klotz concluded from X-ray data[1] that the exterior of a number of proteins is much more hydrophobic than had been assumed. This is in agreement with subsequent findings that at an NaCl concentration of 1 *M* or higher, *i.e.*, under conditions which tend to quench charge effects[2], many proteins are bound by certain agarose-bound amines and that the extent of binding increases with increasing hydrophobicity of the ligand[3–5]. Furthermore, several proteins are bound by hydrophobic adsorbents that, in contrast to the positively charged amino-agaroses[6], carry no charge[7–10] or are neutralized by the presence of a negative charge, *e.g.*, as in the case of agarose substituted with an amino acid[11,12]. Hydrophobic binding actually was found to be stabilized by certain salts, *e.g.*, NaCl[7,13]. These observations are also in accord with the more indirect but overwhelming earlier evidence obtained by Hansch and co-workers on the frequent occurrence of hydrophobic phenomena in biochemical interactions (*e.g.*, see ref. 14). Early observations in this laboratory, discussed in ref. 15, showed that chemically so-called "inert" hydrophobic groups in the substrate often play an important role in enzyme reactions and affect not only the binding process, as reflected in the Michaelis constant (K_m), but also the rate of subsequent breakdown of the enzyme–substrate complex as expressed by the maximal rate (V_m).

All of these findings suggest that hydrophobicity probably plays an important

role in the biological function of proteins and in the binding and transport of metabolites (and drugs). Hydrophobic effects in adsorptive binding involving nucleic acids are also indicated (see ref. 2).

Studies of the interactions of proteins and of other biochemically important structures with adsorbents carrying covalently bound hydrophobic groups are of great importance as "models" for "solid-state" chemical processes occurring *in vivo*[16]. However, under favorable conditions, *e.g.*, in the presence of relatively high concentrations of certain salts, such adsorbents can also be used for the chromatographic separation of proteins based on differences in their hydrophobic properties. The present communication is concerned with the separation of proteins by this means (also see refs. 3, 12, 17). Some preliminary data on another non-ionic but apparently non-hydrophobic parameter (presumably hydrogen bonding) involved in the adsorption of certain proteins, are also presented.

2. EXPERIMENTAL

The *n*-alkylamino-agaroses were prepared via CNBr activation[18,*] from Sepharose 4B or CL-Sepharose 4B (Pharmacia) and either aged for several weeks or heated for 1–2 h at 100° at pH 4–5 before use[19]. The alkylamines were crystallized as the hydrochlorides from ethanol through the addition of ethylether at −15°. DEAE-agarose was obtained by courtesy of BioRad Labs. Before equilibration with the experimental medium, the adsorbents were exhaustively washed with 50% ethylene glycol (EG) or dimethylformamide (DMF) in 0.3–1.0 *M* NaCl. Their relative degrees of substitution were estimated from the extent of irreversible binding of Ponceau S in the absence of salt[17].

Blood serum was dialyzed against and individual proteins were dissolved in the applied medium. In order to prevent excessive packing, the columns were supported, if necessary, with siliconized glass beads of appropriate size[20]. The loaded columns, which were kept at *ca.* 5°, were washed with the ambient medium until, as indicated by the absorbance or fluorescence of the effluent, little or no further material was released, and the bound protein was subsequently eluted by means of 50% EG or 50% DMF in 0.3–1.0 *M* NaCl.

SDS electrophoresis was carried out as described by Weber and Osborn[21] in 10% polyacrylamide gels at *ca.* 3 mA per gel for about 19 h. Before electrophoresis, the salt and the EG or DMF of the eluates were removed by drying the mixture at *ca.* 5° in a dialysis bag placed in a strong solution of polyethylene glycol (Carbowax 4000, Union Carbide), followed by reconstitution with an amount of water sufficient to produce a suitable protein concentration. Experimental details are described in the legends to the Figures.

3. EFFECT OF LIGAND HYDROPHOBICITY AND OF SALT (NaCl) CONCENTRATION ON BINDING OF SERUM ALBUMIN

Fig. 1, reproduced from an earlier publication[16], shows the effect of the hydrophobicity of the ligand and of the concentration of NaCl in the medium on the

* CNBr control: unsubstituted, inactivated, CNBr-treated agarose. C_0: CNBr-activated agarose treated with ammonia. C_{1-8}: agarose substituted via CNBr activation with *n*-alkylamines of varying (1–8) hydrocarbon chain lengths.

extent of binding of serum albumin by agarose carrying a diethylaminoethyl-, *n*-hexylamino-, *n*-heptylamino-, 4-phenylbutylamino(PBA)-, or *n*-octylamino group. Since the alkylamines keep their basic properties in the bound state[6] and, since their p*K* is *ca.* 10 (ref. 9), they are positively charged at the pH values usually employed. Therefore, at low ionic strength the adsorbents behave as ion exchangers and they all strongly bind the negatively charged serum albumin at the applied pH of 8.

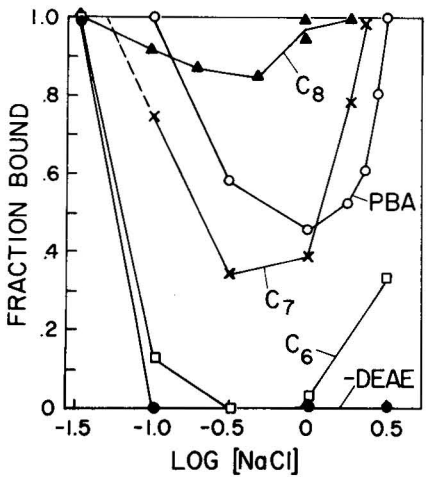

Fig. 1. Effect of salt (NaCl) concentration on the fractional binding of serum albumin by *n*-caprylamino(*n*-C_8)-, 4-phenyl-*n*-butylamino(PBA)-, *n*-heptylamino(*n*-C_7)-, *n*-hexylamino(*n*-C_6)-, or diethylamino-ethyl(DEAE)-agaroses. A few milligrams of the protein were applied to a 1 ml column of an adsorbent equilibrated with 0.01 *M* Tris–HCl (pH 8). The loaded column was washed exhaustively with the buffer alone and subsequently with buffer containing NaCl. Reproduced from previously published results[16] by permission of Marcel Dekker, Inc.

As can be seen (Fig. 1), if the salt concentration is increased, ionic binding begins to be reversed but upon further increase of the salt concentration binding increases again, presumably due to hydrophobic interaction. It should be noted that the hydrophobicity of the phenyl group of PBA is equivalent to that of only 3–4 straight-chain hydrocarbons[22–24].

It is clear that at intermediate, including physiological, NaCl concentrations binding is through a combination of ionic and hydrophobic forces (see also refs. 4, 25, 26). In this region of the salt concentration partial elution will be obtained by raising of the ionic strength *per se*. However, a salt more "chaotropic" than NaCl would be more effective since such a salt may reverse hydrophobic as well as ionic binding. Together with the fact that in this region the binding increases with increasing hydrophobicity of the ligand (Fig. 1), this could erroneously be interpreted as hydrophobic binding that is reversed by the addition of NaCl, whereas in fact the extent of hydrophobic binding increases with increasing NaCl concentration[7,13] and, presumably, only electrostatically bound protein is released (see also ref. 27).

4. CHARGED *VERSUS* UNCHARGED ADSORBENTS

From a physiological point of view, the region of the salt concentration where

hydrophobic as well as ionic binding can occur is the most interesting, but for studies of hydrophobic phenomena *per se*, it is advantageous to separate them from charge effects. For this reason, neutral adsorbents have been prepared (see Introduction). However, for the case of the positively charged amine-substituted agaroses[6], one can carry out the experiments in the presence of high concentrations of a salt such as NaCl, which quenches the charge effect and at the same time enhances hydrophobic bonding. Thus, with the same adsorbent one can study hydrophobic binding at high, ionic binding at low, and combined hydrophobic and charge effects at intermediate salt concentrations.

Another advantage of the charge on the adsorbent-bound amine is that it presents a convenient means for determining the binding capacity of the adsorbent from the amount of a negatively charged dye, such as Ponceau S, that is "irreversibly" bound in the absence of salt[17]. For this purpose, the column is saturated with a salt-free aqueous solution of the dye, followed by washing with water until no further color is released, elution by means of a suitable agent (*e.g.*, 50% EG or DMF in 0.3 *M* NaCl), and spectrophotometric determination of the amount of dye at 525 nm. The evidence[17] indicates that this procedure essentially measures the density of the charged amino group that is introduced together with the hydrophobic group*. An example of the usefulness of the Ponceau procedure is shown in Fig. 2.

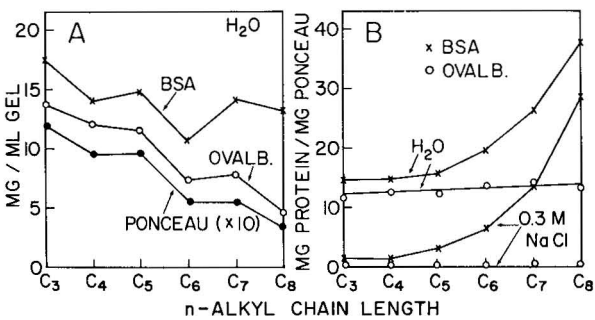

Fig. 2. Effect of C-chain length (C_3–C_8) of *n*-alkylamine-substituted agaroses on their binding capacities for bovine serum albumin (BSA) and ovalbumin (OV) as related to the capacities for binding of Ponceau S in the absence of salt. After saturation of the adsorbents (*ca.* 1 ml) with protein in the presence of 0.01 *M* Tris–HCl (pH 8) and exhaustive washing with water, the columns were first eluted with 10 ml of 0.3 *M* NaCl in 0.01 *M* Tris–HCl, followed by elution with 10 ml of the same solution containing 50% DMF. A, binding capacities of the adsorbents for BSA, OV and Ponceau S in the presence of water without additions. B, binding capacities in water and those in 0.3 *M* NaCl expressed as the amount of protein bound relative to the amount of Ponceau S bound in the absence of salt.

* As was previously noted[4], unsubstituted aged CNBr-treated agarose preparations often are positively charged to some extent. Consequently, they also bind Ponceau S. However, dye-binding of CNBr-treated CL-Sepharose is abolished by preheating the gel for 1 h at 100°. Preliminary results also indicated that heating of the substituted cross-linked agaroses (C_0–C_4) for either one or two hours at 100°, decreased their Ponceau binding capacities by *ca.* 60%, regardless of the degree of substitution (unpublished observations).

5. NORMALIZATION OF PROTEIN BINDING CAPACITIES OF ADSORBENTS

Fig. 2A shows the amounts of bovine serum albumin (BSA), ovalbumin (OV) and Ponceau S bound by a homologous series of *n*-alkylamine-substituted adsorbents under saturating conditions. These data seem rather meaningless until they are plotted as mg protein bound per mg Ponceau *versus* the C-chain length of the adsorbent (Fig. 2B). Plotted in this manner they show that the hydrophobicity of the adsorbent has little or no effect on the binding capacity for OV but that above C_5 the binding of serum albumin is greatly affected. This normalization procedure not only circumvents the effect of differences in the degree of substitution of the adsorbents but also that of slight differences in column size.

The data of Fig. 2B also indicate that electrostatic binding involving the positive charge on the ligand is completely or nearly completely quenched in 0.3 *M* NaCl and that any interaction above this level of the salt concentration is non-ionic. Such binding occurs with BSA but not in the case of OV. Above 0.3 *M* the effect of NaCl can still be studied over a wide range of concentrations without interference of charge effects. Elution can be achieved with the aid of an agent such as EG[3], if necessary, in combination with a limited decrease in ionic strength.

TABLE I

PROTEIN BINDING BY *n*-HEXYLAMINO-AGAROSE IN THE PRESENCE OF 3.3 *M* NaCl

Percent of $\leq$ 5 mg protein held by 25 ml of the adsorbent at *ca.* 5° after washing with 2–3 bed volumes of the medium (3.3 *M* NaCl in 0.01 *M* Tris–HCl, pH 8) and subsequently eluted by 50% EG in buffer containing 1 *M* NaCl.

Protein	*Protein binding (%)*
7S γ-Globulin	100
Serum albumin	100
β-Lactoglobulin	100
Chymotrypsinogen	100
α-Chymotrypsin	$>$ 90
DNase I	$>$ 90
RNase	$\approx$ 75
Cytochrome *c*	$\approx$ 25
α-Lactalbumin	$\approx$ 15
Myoglobin	$<$ 10
Ovalbumin	$<$ 5

6. GENERALITY OF NON-IONIC PROTEIN BINDING

Table I shows the relative extent of binding of a number of arbitrarily chosen proteins by *n*-hexylamine-substituted agarose in the presence of 3.3 *M* NaCl. In contrast to Fig. 2, where small (*ca.* 1 ml) columns were saturated with protein, only a few milligrams of a protein were applied to a 25-ml column.

It can be seen that under the experimental conditions employed, *i.e.*, with relatively small amounts of protein on a rather large column and in the presence of NaCl in high concentration, all or nearly all of the proteins are bound, at least to some extent. The fact that positively charged protein species (*e.g.*, chymotrypsinogen), as

well as negatively charged species (*e.g.*, serum albumin), may be strongly bound indicates the absence of charge effects. This is also indicated by the finding that OV, which like serum albumin is negatively charged, shows little or no binding.

It should be noted that with columns more highly substituted and/or ligands more hydrophobic than *n*-hexylamine, some or all of the proteins that show little binding under the conditions of Table I, may also be extensively bound. For instance, it has been shown that with adsorbents of higher hydrophobicity, even OV which displays little hydrophobicity under the conditions of Table I, is also bound to a considerable extent[28].

In any event, these and other results[17] suggest that hydrophobicity is a general property of proteins that varies from one protein to the next and can thus be applied as an independent parameter for protein separation in addition to other parameters such as electrical charge or molecular size. The advantage of hydrophobic over ionic adsorption chromatography is that the hydrophobic factor is much more selective because widely varying degrees of hydrophobicity can be employed. Qualitatively, for ion exchange the choice is limited to either positive or negative.

7. DETERMINATION OF RELATIVE HYDROPHOBICITIES OF PROTEINS (HYDROPHOBICITY "SCALE")

The data of Table I are sufficient to distinguish the more hydrophobic proteins from the less hydrophobic ones. However, they do not allow for assignment of a relative hydrophobicity value to each individual protein. For this purpose, it is necessary for the more hydrophobic proteins at the top of Table I to determine the relative extent of binding on adsorbents less hydrophobic than hexylamino-agarose. Conversely, for more accurate determination of the hydrophobicities of the proteins at the bottom of Table I more hydrophobic adsorbents are needed.

It should also be ascertained that the observed binding in each case is determined solely by hydrophobic effects and that other non-ionic parameters (*e.g.*, hydrogen bonding, see below) are not involved.

Since the binding of proteins by the adsorbents would be favored by multiple-point attachment (see refs. 16 and 17) the molecular size of the protein would be a factor. It may be noted (Table I) that cytochrome *c*, α-lactalbumin and myglobin, which have relatively low molecular weights (M.W.), also show relatively little binding. Furthermore, the protein with the highest M.W. (7S γ-globulin) shows extremely strong binding. On the other hand, this does not hold true for OV, which shows little or no binding but has an M.W. which is higher than that of the other proteins of Table I, with the exception of 7S γ-globulin and serum albumin. Such a positive relationship between M.W. and extent of binding also seems to be absent for most of the other proteins.

Results from P.-Å. Albertsson's laboratory on the relative strength of binding of several proteins by the monopalmitoyl ester of polyethyleneglycol[29,30] indicated the following relationship for the binding constants of different proteins: BSA $>$ β-LG $>$ myoglobin $>$ ovalbumin $\approx$ chymotrypsinogen A. With the exception of chymotrypsinogen, these results are not unlike our own. Since multiple point attachment to the palmitoylester would be unlikely, this does not appear to be a factor in this case.

In any event, eventually it may be possible to determine exact hydropho-

bicity indices for proteins in general. Such values could be of equal importance as those of other properties such as isoelectric point and molecular weight.

8. PROTEIN SEPARATION ON HYDROPHOBICITY GRADIENTS

It would seem, that with the aid of a column such as the *n*-hexylamino-agarose column of Table 1, separation would be possible by loading it with a protein mixture in the presence of high concentrations of a salt such as NaCl, followed by differential elution, *e.g.*, with a gradient of increasing EG concentrations in combination with a limited decrease in ionic strength (see above). However, in view of possible extremely strong binding, ascribed to multiple-point interaction of the protein with several adsorbent binding sites ("chelation"), application of a mixture to a column of an adsorbent of arbitrarily chosen hydrophobicity may result in binding of the more hydrophobic proteins that is too strong for subsequent elution by a mild eluent, *i.e.*, recovery may not be possible without denaturation (see also refs. 31 and 32). On the other hand, the less hydrophobic proteins may not be held by the column. For this reason, the use of hydrophobicity gradients has been proposed[12]. Such a gradient consists of a series of interconnected columns of increasing hydrophobicity through which the protein mixture is pumped starting with the least hydrophobic one. In this manner, each protein tends to be held by the column that provides the minimum degree of hydrophobicity required for binding. Thus, after extensive washing with the ambient medium, each of the subsequently disconnected columns may be separately eluted with a relatively mild eluant such as EG[3] which even in 50% concentration appears to have little or no irreversible effect on most proteins[33]. A similar procedure for charcoal chromatography has been proposed by Porath[34].

9. SEPARATION OF BLOOD SERUM PROTEINS ON HYDROPHOBICITY GRADIENTS

The data of Fig. 3 show the extent of binding of serum proteins by a hydrophobicity gradient of the C_4, C_5, C_6, C_7 and C_8 adsorbents. It can be seen that relatively little protein is bound by the C_4 column. A peak appears at C_5, followed again by an increase in binding with increasing chain length from C_6 to C_8.

The SDS electrophoresis patterns of the eluates as well as the patterns of the protein solution after (rec) and before (ser) recycling through the gradient, are shown in the insert. Before electrophoresis all of these solutions were dialyzed and dried against polyethylene glycol and reconstituted with water (see Experimental). A control of an untreated solution of human γ-globulins (Fr.II) is also included.

It should be emphasized that the intensity of the electrophoretic bands cannot be compared quantitatively from one gel to another because the eluates were concentrated to different extents based on their A_{280} values, as shown in the curve. For instance, little γ-globulin is seen in the pattern of the C_8 eluate despite the fact that the two corresponding bands of the heavy and light chains are still present in the pattern of the recycled solution and therefore all of the columns must have been in contact with this protein. The reason could be that this solution, because of its high absorbance, was concentrated the least (see legend to Fig. 3). Another possibility is that competition with the albumin is involved.

Although the mobilities of the bands do not correspond exactly to those of the

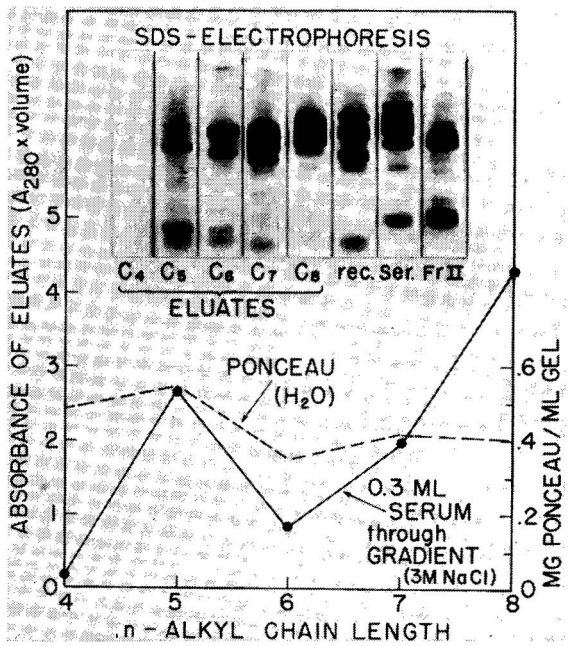

Fig. 3. Adsorption chromatography of 0.3 ml of human blood serum on a hydrophobicity gradient consisting of interconnected 1 ml columns of *n*-butyl(C_4)-, *n*-pentyl(C_5)-, *n*-hexyl(C_6)-, *n*-heptyl(C_7)- and *n*-octyl(C_8)amino-agaroses prepared via CNBr activation from CL-Sepharose 4B. The adsorbents, preheated for 1 h at 100° and pH 4–5, were equilibrated at *ca.* 5° with 3 *M* NaCl in 0.01 *M* Tris–HCl (pH 8). The serum, diluted 1:50 with and dialyzed against the buffer–salt medium, was recycled about ten times through the gradient in the direction of increasing hydrophobicity. This was followed by exhaustive washing with the ambient medium and subsequent elution of the disconnected columns by means of 50% ethylene glycol in buffer containing 0.3 *M* NaCl (see Experimental). Before electrophoresis the eluates of the C_4, C_5, C_6, C_7 and C_8 adsorbents were desalted and concentrated approximately 10-, 4-, 10-, 7-, and 2.5-fold, respectively (see Experimental).

controls (ser, Fr.II), the data indicate that the major component of the protein fraction bound by and eluted from the C_5 adsorbent, as opposed to the C_6, C_7, and C_8 adsorbents, consists of γ-globulin, and that this adsorbent, in contrast to the more hydrophobic ones, binds little or no albumin. Other bands, relatively stronger than those on the Fr.II control, can also be noted.

In any event, separation of the two major serum protein fractions *i.e.*, albumin and γ-globulin, seems to be possible by means of the pentylamine-substituted agarose (see also ref. 8). Similar results have been previously obtained by means of phenylalanine-substituted agarose[11,12]. It is of interest that the hydrophobicity of the phenylalanine side-chain corresponds to that of 4–5 straight-chain hydrocarbons[22,24].

The spilling over of an abundant protein, such as albumin in the case of serum, onto columns more hydrophobic than required for binding of that protein, may defeat the purpose of the hydrophobicity gradient and, in fact, could render these columns useless. The results of Fig. 3 indicate that this is likely to occur in attempts to isolate minor components from whole serum by hydrophobic gradient chromatography on columns more hydrophobic than butylamino-agarose. Therefore, the run shown in Fig. 4 was carried out with less hydrophobic adsorbents. In order to allow minor

components to accumulate, columns four times larger were used and 5 ml instead of 0.3 ml serum was applied. Also, the degree of substitution of the C_4 adsorbent, as indicated by the Ponceau values, was approximately twice as high as that of the C_4 column of Fig. 3. Thus potentially much more protein could accumulate on this column. As indicated by A_{280}, it can indeed be seen that more than 100 times as much was bound. However, the amount rapidly decreases with decreasing C-chain length of the adsorbent (Fig. 4).

The surprising finding is that the CNBr-control binds more than the C_0, C_1 and C_2 and about the same as the C_3 column. A striking feature of the electrophoretic patterns of Fig. 4 is that those of the eluates of the C_0, C_1 and C_2 columns are about the same and that corresponding bands also occur in the eluates of the C_3 and C_4 columns. This indicates that the binding of some of the material in serum is not dependent upon the hydrophobicity of the adsorbent. On the other hand, the CNBr-control as well as the C_3 and C_4 gels seem to contain components that do not occur on the other adsorbents. For C_3 and C_4 this can be ascribed to increased hydro-

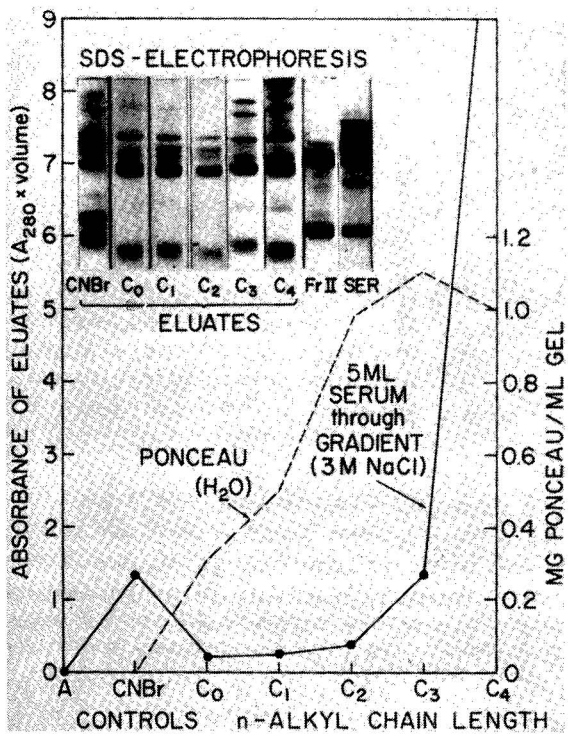

Fig. 4. Adsorption chromatography of 5 ml of human blood serum on a hydrophobicity gradient consisting of *ca.* 4-ml columns of preheated CL-agarose (A), CNBr-activated CL-agarose (CNBr) and CNBr-activated CL-agarose treated with ammonia (C_0) or with methyl (C_1)-, ethyl(C_2)-, *n*-propyl (C_3)- or *n*-butylamine(C_4). The experimental conditions were generally the same as those for Fig. 3, except that the serum was diluted only ten-fold. Before electrophoresis (insert) the eluates of the CNBr, C_0, C_1, C_2, and C_3 adsorbents were desalted and concentrated approximately 8-, 30-, 30-, 20-, and 8-fold respectively. The C_4 eluate was similarly treated but reconstituted to its original volume (see Experimental).

phobicity, but the protein bound by the CNBr-control, as well as the bands common to all these gels, may be due to another type of binding, most likely involving hydrogen bonds (see below).

In any event, if a component appears on a particular column and not on subsequent columns of the series, this indicates that such a component, occurring in trace amounts in the serum, has accumulated on that particular column which apparently provides the minimum degree of affinity required for binding. This is the case, for instance, for some components on the CNBr-control, on the C_3 and possibly on the C_4 column.

Another striking feature of these patterns is that there are two bands, similar to those of the light and the heavy chain of γ-globulin, that occur in all of the electrophoretic patterns, suggesting that all of the corresponding columns bind γ-globulin. This is confirmed by the results of Fig. 5.

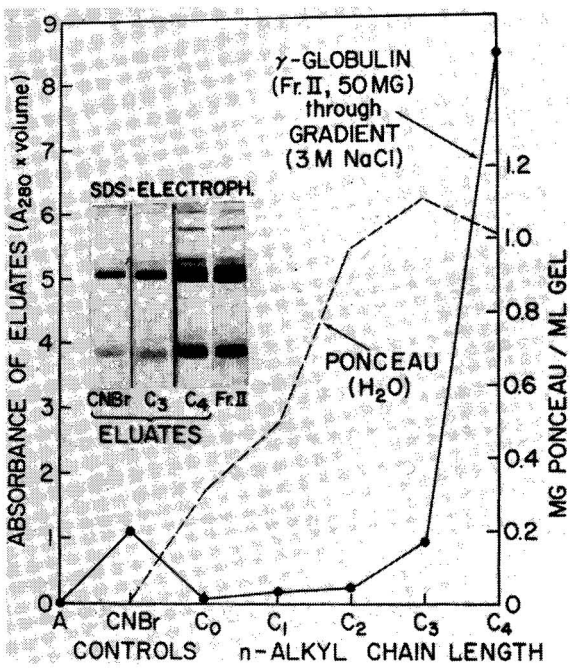

Fig. 5. Adsorption chromatography of 50 mg of "γ-globulin" (blood serum Fr. II), dissolved in 50 ml medium, under the same conditions and on the same columns as those of Fig. 4. For electrophoresis the eluates of the CNBr and C_3 adsorbent were concentrated approximately ten-fold. The C_4 eluate was not concentrated (see legend to Fig. 4).

10. NON-IONIC NON-HYDROPHOBIC BINDING OF γ-GLOBULIN COMPONENT(S)

The shape of the absorbance curve of Fr.II (Fig. 5) strongly resembles that of the whole serum of Fig. 4, suggesting that the curve for whole serum is largely determined by the γ-globulin. The electrophoretic patterns of the proteins bound by CNBr control and by the C_3 and C_4 adsorbents (Fig. 5) seem to be qualitatively identical to the Fr.II control in every detail. This indicates that the same protein unit or units are

bound by all of these gels. As one could expect, in view of the numerous antigenic compounds that can be bound by γ-globulin, this protein in particular should be capable of binding in various ways, *e.g.*, hydrophobically by *n*-alkyl groups larger than C_3 and perhaps by hydrogen-bonding in the case of the CNBr control column and to a lesser extent by the substituted columns as well.

That this binding of γ-globulin by the CNBr control is predominantly non-hydrophobic is also indicated by the data of Fig. 6 which show that very little reversal is obtained by lowering the salt concentration from 3 to 0.3 *M*. On the other hand, binding by the C_4 column is to a large extent reversed by lowering the salt concentration indicating a much larger hydrophobic effect. This holds true for Fr.II (which is mostly γ-globulin) as well as for a preparation of 7S γ-globulin.

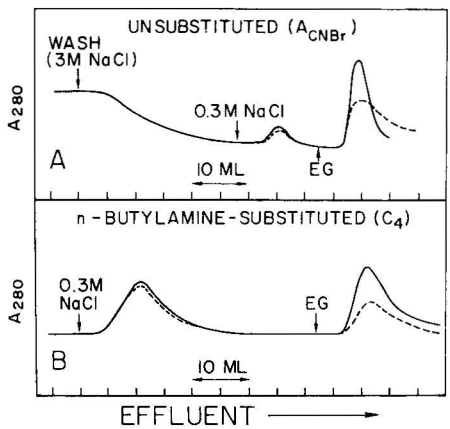

Fig. 6. Elution profiles of 7S γ-globulin (—) and of "Fraction II" (— — —) peaks only, after adsorption on 3–4 ml columns of the CNBr and the C_4 adsorbents (see Fig. 5). Protein solution (10 ml) with an A_{280} value of 0.3–0.4 were recycled several times through a column until the absorbance of the effluent was constant. The column was then washed with 3 *M* NaCl (shown only in the upper part), subsequently with 0.3 *M* NaCl and finally with 50% ethylene glycol (EG) in 0.3 *M* NaCl. Arrows indicate the points of change of medium.

Fig. 6 also indicates that the non-hydrophobic type of binding, like the hydrophobic type, is readily reversed by 50% EG. If the binding by A_{CNBr} is indeed mostly through hydrogen bonding, this could have a bearing on the theory[35] that hydrophobic bonding ultimately depends on the intermolecular structure of water which, in turn, also depends on hydrogen bonding.

It should be noted that the binding capacity of the CNBr column of Fig. 5 for γ-globulin is not more than *ca.* 0.2 mg protein per ml of the settled gel. It would be of interest, therefore, to test other adsorbents with potentially stronger hydrogen bonding capabilities.

11. ACKNOWLEDGEMENTS

This work was supported by U.S. Public Health Service Grants GM22545 and RR05513, by Santa Clara County United Way, and by the Harvey Bassett Clarke Foundation.

12. SUMMARY

Evidence is presented indicating that protein separation by adsorption chromatography, based on differential non-ionic interaction with immobilized hydrophobic ligands, potentially is as generally applicable as ion-exchange chromatography. A procedure for the normalization of binding capacities of amine-substituted agaroses has been presented. Attempts have been made at the separation of proteins in normal human blood serum by means of hydrophobicity gradients consisting of series of interconnected columns of *n*-alkylamino-agaroses of increasing hydrophobicities and equilibrated with 3 *M* NaCl. The γ-globulin fraction, or components thereof, can be bound hydrophobically as well as through another type of salt(NaCl)-stable but non-hydrophobic binding.

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MULTIVALENT INTERACTION CHROMATOGRAPHY AS EXEMPLIFIED BY THE ADSORPTION AND DESORPTION OF SKELETAL MUSCLE ENZYMES ON HYDROPHOBIC ALKYL-AGAROSES

HERBERT P. JENNISSEN

Institut für Physiologische Chemie der Ruhr-Universität Bochum, Postfach 10 21 48, D-4630 Bochum 1 (G.F.R.)

CONTENTS

1. INTRODUCTION

If the degree of substitution of Sepharose 4B with α-aminoalkanes (*i.e.*, alkyl-residue density) is increased, the binding capacity of the gels for proteins is a sigmoidal function of the alkyl-residue density[1]. This correlation has been shown at low ionic strength (μ = 0.03–0.04)[1,2] for the enzymes phosphorylase *b*, phosphorylase kinase, phosphorylase phosphatase, glycogen synthetase, cAMP-dependent protein kinase and other skeletal muscle proteins. Similarly, a sigmoidal relationship between the binding capacity of substituted gels for phosphorylase *b* and the alkyl-residue density could be demonstrated at high ionic strength[3]. An analysis of these positive cooperative binding curves according to Hill[3–5] has led to the conclusion that the proteins described (see above) are multivalently adsorbed on at least 3–9 binding sites (multivalent interaction chromatography[6]). The minimum number of the sebinding sites appears to be temperature dependent[6].

From equilibrium binding studies of phosphorylase *b* on alkyl-Sepharoses of a specific residue density, negative cooperative ligand binding has been concluded[5]. Recently, it was reported that negative cooperativity of phosphorylase *b* binding to butyl-Sepharose might also be inferred from kinetic studies[6].

In this paper, the evidence that strongly indicates that proteins are multivalently adsorbed to alkyl-Sepharose 4B (1–4 carbon atoms) and the practical consequences for chromatographic procedures are presented. An attempt is also made to introduce a systematic order into the diverse nomenclature of chromatography on substituted carbohydrate gels on the basis of the valence involved in the adsorption of the protein ligand.

2. MATERIALS AND METHODS

The preparation of ^{14}C-labelled alkyl derivatives of Sepharose 4B has been described by Jennissen and Heilmeyer[1] and Jennissen[2]. Phosphorylase *b* (*ca.* 80 U/mg) was prepared and freed of AMP according to Fischer and Krebs[7]. All calculations are based on a subunit molecular weight of 100,000 (ref. 8). The activity was measured according to Haschke and Heilmeyer[9]. Unless otherwise stated, the capacity of the gels was determined at apparent ligand-matrix equilibrium (see the isotherms below). All experiments were performed in thermostated Plexiglas beakers (2.5 cm I.D. × 9 cm) employing a 1.5-cm magnetic stirring bar as described by Jennissen[5]. The isotherms (for a detailed description, see ref. 5) were determined by incubating increasing concentrations of phosphorylase *b* with butyl-Sepharose in buffer containing 10 m*M* tris(hydroxymethyl)aminomethane/maleate, 5 m*M* dithioerythritol, 1.1 *M* ammonium sulphate and 20% sucrose (pH 7.0) for 30 min at a stirring rate of *ca.* 700 rpm (ref. 3). The amount of phosphorylse *b* adsorbed on the gel was calculated from the difference between the values of the initial and the final free phosphorylase *b* concentration (activity measurements) after 30 min of adsorption employing unsubstituted Sepharose 4B under identical conditions as the control.

The method for the measurement of the kinetics of the desorption of phosphorylase *b* in the above buffer was described by Jennissen[6]. For the sampling procedure, see ref. 5.

3. RESULTS AND DISCUSSION

3.1. Influence of substitution procedures on the substituted matrix

3.1.1. Gel contraction and the degree of substitution

The degree of substitution is usually measured on a volume basis in micromoles of substituent per millilitre of packed Sepharose[1]. The alkyl-residue density can, however, also be expressed on a weight (moles of substituent per gram of dry agarose)[10] or molar basis (moles of substituent per mole of anhydrodisaccharide, see below). Låås[10] has shown that a drastic decrease in gel volume (50–60%) of benzylated Sepharose CL-2B may occur at substitutions of 55–110 μmole/ml gel. Therefore, large discrepancies in the value of the residue density could occur, depending on the units employed. It will be shown below (see Table 2) that under the conditions employed here[1–3] a maximal volume decrease of *ca.* 10% is found for a substituted

butyl-Sepharose containing 44 μmole/ml packed gel. This volume change has virtually no influence (see below) on the evaluation of binding data. Nevertheless, as changes in gel volume do occur, it is probably more appropriate to express the substituent density for binding site determinations on a molar (or weight) basis. However, then it is assumed that a gel contraction does not lead to a higher density of the residues on the agarose matrix.

3.1.2. Uniform distribution of residues and substitution procedures

In the described coupling procedure (see Section 2) the reactive sites are introduced by varying the concentration of cyanogen bromide (see Table 2) in the reaction mixture. The coupling to the alkylamine[11] occurs at a constant (maximal concentration change 0.3%)[1] and very high amine concentration (2 *M*). It is therefore unlikely and can be excluded[1] (see Table 2) that the introduction of alkyl residues is a sigmoidal (see the implications below) function of the initial cyanogen bromide concentration. A uniform substitution of Sepharose by the cyanogen bromide method is also indicated by the electron microscopy of ferritin coupled to Sepharose 6B[12].

In contrast, if an alkyl halide is reacted directly with non-activated Sepharose CL-6B[13] the degree of substitution increases as a sigmoidal function of the dilute and limiting initial concentration of the halide in the mixture. This behavior has been interpreted as indicating an uneven (cooperative) distribution of alkyl residues on the matrix[13].

3.2. Positive cooperative binding as a function of the alkyl-residue density

3.2.1. Low ionic strength (e.g., μ = 0.03–0.04)

In Table 1, the data so far reported on the positive cooperative adsorption of enzymes on alkyl-Sepharoses at low ionic strength are summarized.

In a similar manner to Freundlich isotherms (see negative cooperativity below), the sigmoidal curves resulting from a plot of the adsorbed amount of ligand per unit of packed Sepharose against the degree of gel substitution with alkyl residues can easily be analysed according to the power function of Freundlich[4,5,15]:

$$\log a = \log \alpha + 1/n \log c \tag{1}$$

where a (also called binding capacity[1]) is the amount of ligand adsorbed (*e.g.*, in milligrams per millilitre of packed gel), α is the adsorption constant (*e.g.*, in milligrams per millilitre of packed gel), $1/n$ is the adsorption exponent and c is either the alkyl-residue density (*e.g.*, in micromoles per millilitre of packed gel, see Table 1) or the free ligand equilibrium concentration (*e.g.*, in milligrams per millilitre, see Table 3). The adsorption exponent (c = alkyl-residue density) corresponds to the initial increment of the sigmoidal, positive cooperative binding curves when these are plotted in double logarithmic coordinates.

If saturation is approximated, analysis according to Scatchard[16] is feasible. With the extrapolated saturation value of the Scatchard plot, the corresponding Hill plot[5,17] can be obtained for analysing the positive cooperativity. The calculated Hill coefficient (n_H), which is larger than unity, is an indication of the minimum number of binding sites involved in adsorption[5,18]. This type of ligand adsorption, which ideally

TABLE 1

CONSTANTS OF THE FREUNDLICH AND THE HILL EQUATION OF THE BINDING OF SKELETAL MUSCLE ENZYMES TO ALKYL-SEPHAROSES AS A FUNCTION OF THE DEGREE OF SUBSTITUTION AT LOW IONIC STRENGTH

The original experimental data employed in the calculations are given in the corresponding reference. For definition of $1/n$, n_H, $K_{0.5}$ and packed Sepharose, see ref. 5 and the text. The enzymes were adsorbed from crude muscle extracts[1] from either mixed, white or red muscle[14]. Saturation of the gels with phosphorylase kinase was extrapolated from Scatchard plots to be: mixed muscle 1.0 (methyl), 1.4 (ethyl), 1.8 (butyl); white muscle 0.5 (methyl); and red muscle 0.16 (methyl) mg/ml packed gel, respectively, assuming a specific activity of the pure enzyme to be 8500 units/mg. For further details see the text, Tables 3 and 5 and the references.

Enzyme	*Alkyl residue*	$1/n$	n_H	$K_{0.5} \cdot 10^{-1}$ (*l packed Sepharose/mole*)	*Reference*
Phosphorylase Kinase					
mixed muscle	Methyl	5.8	6.7	5.5	1
	Ethyl	3.6	3.8	6.8	
	Butyl	3.5	3.7	8.2	
Fast twitch muscle (white)	Methyl	7.5	9.2	6.8	
Slow twitch muscle (red)	Methyl	7.8	8.7	7.9	2
Phosphorylase Phosphatase					
Fast twitch muscle (white)	Methyl	2.9			2
Slow twitch muscle (red)	Methyl	3.6			
Phosphorylase *b*					
mixed muscle	Ethyl	8.5			1
	Butyl	4.8			

corresponds to an "all-or-none" model, has been called imperative multivalent binding[3].

With phosphorylase kinase (Table 1), where both the adsorption exponent and the Hill coefficient have been calculated, the value of the Hill coefficient is consistently 1.1–1.2-fold higher than the adsorption exponent. A similar relationship has been shown for negative cooperative binding[5] (see below). One can therefore conclude that, in those instances when a Hill coefficient cannot be calculated (*e.g.*, phosphorylase phosphatase and phosphorylase *b*) because saturation is not obtained, the adsorption exponent gives a good approximation of a minimum value of the Hill coefficient. For the adsorption of phosphorylase kinase on methyl-agarose, the Hill coefficients indicate that at least 7–9 binding sites are necessary. There appears to be no difference in the number of binding sites essential for the two isoenzymes[14] from fast and slow twitch muscle. This may not be true for phosphorylase phosphatase from these two types of muscle.

If the chain length of the alkyl residue is increased from methyl to ethyl and butyl, the adsorption exponents and the corresponding Hill coefficients (n_H) decrease from 6.7 to 3.8 and 3.7, respectively. A similar decrease in the adsorption exponent is observed for phosphorylase *b* adsorption on ethyl- ($1/n = 8.5$) and butyl-Sepharose ($1/n = 4.8$). It has therefore been concluded[4,5] that the number of binding sites necessary for adsorption of a ligand molecule decreases as a function of the alkyl-

residue chain elongation. Therefore, if the chain length is sufficiently great and the interaction of a single alkyl residue with the protein yields the free energy necessary for adsorption, the Hill coefficient should approach unity and a non-cooperative relationship between the gel capacity and the alkyl-residue density is to be expected. This, however, does not exclude multiple contacts between a single residue and the protein ligand. Such a binding behaviour of low residue cooperativity appears to have been observed on uncharged alkyl derivatives of Sepharose[13,20] (see Section 3.1.2.).

The apparent association constant of half-maximal saturation ($K_{0.5}$; Tables 1 and 5) corresponds to the reciprocal of the alkyl-residue density at half-maximal saturation with ligand. With phosphorylase kinase it can be calculated from these constants (see Table 1) that the apparent alkyl-residue density essential for half-maximal saturation decreases from 18.2 to 14.7 and 12.2 μmole/ml packed Sepharose for the methyl, ethyl and butyl derivative, respectively (see Table 5).

3.2.2. High ionic strength ($\mu > 1$)

Various studies[21,22] have indicated that ionic groups can be involved in the adsorption of proteins on alkyl derivatives of cyanogen bromide-activated Sepharoses. These interactions should be reduced or eliminated at high ionic strength. Recently, it was shown[5] that the adsorption of phosphorylase *b* on butyl-Sepharose in the presence of 1.1 *M* ammonium sulphate is in fact an endothermic reaction. Therefore, it was pertinent to show that the multivalent interactions observed at low ionic strength (Table 1) can also be detected in the case of truely hydrophobic (*i.e.*, endothermic) adsorption of proteins on alkyl-Sepharoses.

Therefore, a new series of Sepharose derivatives of increasing degree of substitution was synthesized (Table 2). From the data, it can be calculated that the volume of the gel substituted with 44 μmole/ml packed gel has decreased by *ca.* 10%. The parameters calculated from binding studies are not significantly different whether the butyl-residue density is expressed on a volume or a molar basis (compare the results of Jennissen[6] with Fig. 1). This also demonstrates that the previous evaluations (Table 1) are soundly based.

TABLE 2

SUBSTITUTION OF CYANOGEN BROMIDE-ACTIVATED SEPHAROSE 4B WITH BUTYL RESIDUES

The absolute dry weight of the substituted agarose gels[5] was employed for the calculation of moles of anhydrodisaccharide (molecular weight 306). For the butyl derivative (CH_3–CH_2–CH_2–CH_2–N(–)–C(–)) a molecular weight of 83 was employed. For further details see the text, Section 2 and ref. 1.

BrCN (mg/ml)	*Degree of substitution*	
	μMoles/ml packed gel	*Moles butyl residue per mole anhydrodisaccharide*
1	3.8	0.038
2	6.2	0.062
4	12.1	0.121
8	20.6	0.204
15	29.9	0.308
30	44.0	0.401

In the data presented previously for low ionic strength (Table 1), the capacity of the gels was measured by a column technique[1,2] under the assumption of high binding constants (see $K_{0.5}$ in the paper by Jennissen[5] and below) and low ligand leakage during the column wash. These assumptions can be neglected if the gel capacity is measured at apparent ligand-matrix equilibrium and if the gel capacity of the various gels is compared at identical equilibrium, free ligand concentrations[3,19]. These conditions are most easily met if adsorption isotherms are performed for each gel under study. The adsorption constants (α) and the adsorption exponents ($1/n$) of the Freundlich isotherms are listed in Table 3. The adsorption constants characteristically increase and reach a plateau while the adsorption exponents decrease as a function of the butyl-residue density.

TABLE 3

CONSTANTS OF THE FREUNDLICH ISOTHERMS OF THE BINDING OF PHOSPHORYLASE *b* TO BUTYL-SEPHAROSES AS A FUNCTION OF THE FREE PHOSPHORYLASE *b* EQUILIBRIUM CONCENTRATION IN THE PRESENCE OF 1.1 *M* AMMONIUM SULPHATE

The adsorption constant (α, mg/ml packed Sepharose) and the adsorption exponent ($1/n$) were calculated by the least-squares method (eqn. 1) from the isotherms. For further details, see the legend to Fig. 1, Table 1, methods, the text and ref. 5.

Temperature (°C)	*μMoles/ml packed gel*	α	$1/n$
5	3.8	1.1	0.95
	6.2	3.7	0.72
	12.1	12.2	0.55
	20.6	22.6	0.54
	29.9	21.4	0.46
	44.0	24.7	0.52
34	3.8	2.5	0.95
	6.2	4.6	0.68
	12.1	18.9	0.57
	20.6	34.7	0.52
	29.9	34.6	0.43
	44.0	38.2	0.42

From the isotherms (Table 3) the isosteric heats of adsorption (ΔH) at a capacity of $a = 1.5$ mg of phosphorylase *b* per millilitre of packed gel can be calculated to lie between 2 and 13 kcal/mole (for method, see ref. 5). This demonstrates the endothermic nature of phosphorylase binding on all butyl-Sepharoses (Table 2) independent of the alkyl-residue density. In Fig. 1A, the capacity of the butyl-agaroses for the ligand phosphorylase *b* (calculated from the isotherms in Table 3 at an apparent equilibrium concentration of 0.07 mg/ml) is plotted as a function of the butyl-residue density. Sigmoidal curves result which, when re-plotted according to Scatchard, are concave downwards (see insert in Fig. 1A). An increase in temperature from 5° to 34° leads to an increase in the capacity of the gel (see ΔH above). Fig. 1B shows the corresponding Hill plots (*ca.* 1–90% saturation). An increase in temperature from 5° to 34° decrease the Hill coefficient from 3.6 to 3.0. The apparent association constant of half-maximal saturation is reduced from 7.3 to 6.0 mole of anhydrodisaccharide

per mole of butyl residue (see also ref. 6). It can therefore be concluded that an increase in temperature from 5° to 34° leads to a decrease in the minimum number of binding sites (from 4 to 3, *i.e.*, nearest integers of the Hill coefficients) necessary for the adsorption of a phosphorylase *b* molecule. Correspondingly, the slight decrease in the $K_{0.5}$ value indicates a decrease in affinity as a result of the temperature enhancement.

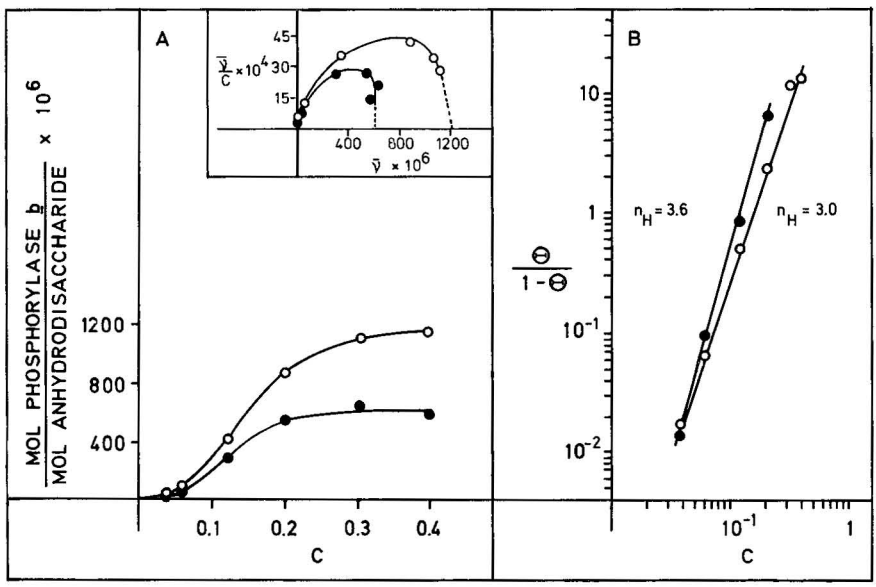

Fig. 1. (A) Adsorption of phosphorylase *b* in the presence of 1.1 *M* ammonium sulphate at 5° (●) and at 34° (○) as a function of the butyl-residue density (equilibrium concentration *ca.* 0.07 mg/ml). Insert: Scatchard plots of the sigmoidal binding curves. $\bar{v}$ denotes the gel capacity for phosphorylase *b* in moles of enzyme per mole of anhydrodisaccharide. *C* is the alkyl-residue density in moles of residue per mole of anhydrodisaccharide. The broken lines indicate the mode of extrapolation. (B) Hill plot of the adsorption of phosphorylase *b* as a function of the butyl-residue density under the conditions described in (A). The fractional saturation of the gel (θ) was calculated from the extrapolated value of 610 and 1220 μmole of phosphorylase *b* per mole of anhydrodisaccharide at 5° and 34°, respectively, as shown in the Scatchard plot in (A). n_H is the Hill coefficient. The apparent association constants of half-maximal saturation ($K_{0.5}$) are 7.3 and 6.0 mole of anhydrodisaccharide per mole of butyl residue at 5° and 34°, respectively. For further details, see the text and ref. 5.

The following simplified model may explain these results. For example, at 5° twelve alkyl residues corresponding to twelve *binding sites* are capable of binding three molecules of phosphorylase *b*. Each molecule of the enzyme covers the minimum number of four binding sites, which are called a *binding unit*. As one binding unit contains four binding sites, twelve binding sites are capable of binding three molecules of ligand. If the temperature is increased to 34°, one binding unit now contains only three binding sites. Thus twelve binding sites are now capable of binding four rearranged molecules of the enzyme. The temperature increase apparently leads to an enhancement of the free energy of a single hydrophobic interaction. Therefore, at the higher temperature apparently only three binding sites are now necessary for the adsorption of one phosphorylase *b* molecule. However, the affinity of the binding unit

containing three binding sites appears nevertheless to be lower than that of the binding unit containing four binding sites. One would therefore expect that the described temperature rise would result in a capacity ratio of 4:3 for the gel capacities at 34° and 5°, respectively. Experimentally, the gels show a capacity ratio of *ca.* 4:2.4, which is higher than the model predicts. This difference may be due to the simplifications involved.

3.3. Negative cooperativity as a function of fractional ligand saturation of the gel

3.3.1. Low ionic strength

Equilibrium binding studies of the exothermic adsorption of phosphorylase *b* on methyl- and butyl-agaroses (Freundlich isotherms) at low ionic strength yield non-linear Scatchard plots (curved concave upwards) and Hill coefficients below unity[5]. This behaviour has been interpreted as a decrease in ligand affinity as a function of fractional saturation (negative cooperativity) on a qualitatively heterogeneous (*e.g.*, ionic and hydrophobic binding sites) lattice of binding sites[5].

3.3.2. High ionic strength

Similar isotherms have been obtained for phosphorylase *b* in the presence of 1.1 *M* ammonium sulphate[5] (see Table 3) and have led to the same interpretation as indicated above. From the positive heats of adsorption (see ΔH above) it has been concluded that this reaction is entropy driven and therefore may occur on a qualitatively homogeneous (hydrophobic binding sites) lattice of binding sites[5]. The observed decrease in affinity (negative cooperativity) is probably due to a number of factors[5], *e.g.*, sequential adsorption, competition of ligands, binding-unit overlap (together often termed non-independent binding) and steric factors such as configurational entropy. These factors are linked to the basic multivalent interactions between matrix and ligand which underly the above effects.

3.3.3. Kinetics of desorption

The rate of [^{3}H]phosphorylase *b* desorption during incubation of butyl-Sepharose (20.6 μmole/ml packed gel) pre-loaded with reduced [^{3}H]phosphorylase *b*

TABLE 4

DESORPTION KINETICS OF TRITIUM-LABELLED PHOSPHORYLASE *b* ON BUTYL-SEPHAROSE

The desorption experiments were performed after 20-fold dilution of the gel in the presence of 1.1 *M* ammonium sulphate at 5°. The apparent, initial desorption rates in the presence and absence of unlabelled, reduced phosphorylase *b* (Cold phos) was calculated between 0 and 2 min of desorption. The specific radioactivity of the labelled enzyme was *ca.* 10^5 cpm/mg. For a description of the experimental procedure, see Section 2, the text and refs. 6 and 23.

Gel load (mg [^{3}H]phosphorylase/ml packed gel)	*Initial rate of desorption (μg [^{3}H]phosphorylase/ml packed gel × min)*		*Stimulation (n-fold)*
	Control	*+Cold phos*	
2.0	16	87	5.44
4.5	48	118	2.46
16.5	393	345	0.88

in buffer without and with *ca.* 1 mg/ml of non-labelled, reduced enzyme, respectively, is shown in Table 4. In the presence of non-labelled phosphorylase *b* the initial desorption rate is stimulated up to 5-fold (Table 4). This enhancement of the desorption rate decreases as a function of fractional saturation of the gel and is absent if the gel is pre-loaded with *ca.* 16 mg of [^{3}H]phosphorylase *b*/ml packed gel (*ca.* 50% fractional saturation).

These preliminary results may be explained by the tentative model[23] that the binding of a non-labelled phosphorylase *b* molecule to a matrix containing adsorbed ^{3}H-labelled enzyme leads to a decrease in the affinity of the matrix for the ^{3}H-labelled enzyme molecule. This decrease in affinity would be manifested by an enhanced initial desorption rate. Hence these kinetic results would be in agreement with negative cooperativity of ligand binding. Similar observations have been reported for the binding properties of insulin receptors[31]. The kinetic data are also in agreement with the proposed model of multivalent effector–receptor interactions[5,6,23].

3.4. Practical consequences of multivalent interactions in the optimization of chromatographic procedures on alkyl-agaroses

3.4.1. Optimal alkyl-residue density

The binding affinity of the matrix is a result of the simultaneous interaction of a critical number of multiple sites (depending, for example, on the alkyl-residue density) and the affinity of a single binding site (depending, for example, on the number of carbon atoms per residue). To purify a protein, one must therefore determine the optimal density and the optimal chain length of an alkyl residue for the adsorption and elution of the ligand. How this can be done is illustrated for the enzyme phosphorylase kinase which is adsorbed from a crude muscle extract at low salt concentrations on alkyl-Sepharoses[1] containing a heterogeneous lattice of binding sites (Table 5).

The chromatographic optimum of the listed gels for phosphorylase kinase at low ionic strength (Table 5) has been reported by Jennissen and Heilmeyer[1]. If the specific activity of this enzyme in the adsorbed state (calculated from the total enzyme activity and the total protein adsorbed per millilitre of packed gel) is plotted against the alkyl-residue density, a maximal specific activity is obtained at the optimal alkyl-residue density. These gels therefore exhibit a certain binding specificity as the maximal specific activity of the enzyme adsorbed to the gel is *ca.* 15-fold higher than in the crude extract (see Table 5). It is evident from Table 5 that this optimal density compares quite well with the reciprocal of the apparent association constant of half-maximal saturation ($K_{0.5}$; compare the values given in Tables 1 and 5). The optimum may in fact be identical with this value, but gels containing this degree of substitution, $(K_{0.5})^{-1}$, were not obtained. As the maximal specific activity of the enzyme on the gel correlates with the purification factor obtained (12–18-fold from a crude extract, yield 80–98%; see Table 5), it can be concluded that irrespective of the residue chain length the three gels are equally efficient for the purification of phosphorylase kinase provided that an alkyl-residue density of half-maximal saturation is employed and the salt concentration for elution is increased.

Optimization of a gel for the purification of a new protein would therefore commence by testing one or two gels of intermediate hydrophobicity (3–6 carbon

TABLE 5

OPTIMIZATION OF THE MULTIVALENT INTERACTION CHROMATOGRAPHY OF PHOSPHORYLASE KINASE ON ALKYL-SEPHAROSES

The specific activity is calculated for the enzyme in the adsorbed state. Recovery is expressed as that percentage of total enzyme units adsorbed (100%) which can be eluted by a given salt concentration. The specific activity of phosphorylase kinase in the crude extract prior to adsorption was 113, 86 and 105 units/mg for the experiment employing the methyl, ethyl and butyl derivative, respectively. For further details and definitions, see Table 1, the text and ref. 1.

Sepharose derivative	$(K_{0.5})^{-1}$ *(μmole/ml packed gel)*	*Chromatographic optimum*			
		Residue density (μmole/ml packed gel)	*Specific activity (units/mg)*	*Elution with NaCl*	
				[NaCl] (M)	*Recovery (%)*
Methyl	18.2	20.7	1790	0.05	68
				0.12	98
Ethyl	14.7	19.2	1320	0.12	47
				1.0	77
Butyl	12.2	15.6	1460	1.0	84

atoms) from a homologous series (series I) of alkyl-Sepharoses to elucidate which alkyl derivative is capable of adsorbing the protein at all. Then a second series (series II) of alkyl-Sepharoses of increasing alkyl-residue density is synthesized. A sufficiently long hydrocarbon chain (series I) should be employed so that a saturation of the binding capacity of the gel as a function of the residue density can be expected. (For examples of enzymes where saturation is not yet obtained at short alkyl-residue chain lengths, see phosphorylase *b* and phosphorylase phosphatase in Table 1.) The Hill plot of the binding data then yields the minimum number of binding sites (n_H) and the optimal alkyl-residue density (calculated from $K_{0.5}$).

3.4.2. Fractional saturation of gel with protein ligand

As has been shown above, the affinity of an alkyl-Sepharose matrix decreases as a function of the fractional saturation with ligand. Therefore, high equilibrium concentrations of ligand do not lead to the expected, proportional increase in capacity. Furthermore, enzyme leakage from the gel will be observed at high fractional saturation. Efficient adsorption is therefore obtained at relatively low enzyme concentrations (crude extracts) where the apparent, high affinity sites are preferentially employed. From Table 4 and the conclusion of binding unit overlap (see Section 3.3.3), one ligand molecule can displace another on alkyl-Sepharoses. This effect at high concentrations of displacing proteins is highest at very low (*e.g.*, 1–5%) fractional saturation (Table 4). In general, therefore *ca.* 15–20% fractional saturation of a gel with protein for a chromatographic procedure appears optimal (for the role of hysteresis see ref. 23). A decrease in affinity with fractional saturation and competitive displacement of ligand by contaminating proteins also make it understandable that the maximal purification of a protein from a crude extract by multivalent interaction chromatography is in general no higher than 10–30-fold (*e.g.*, ref. 1).

3.4.3. Elution

The desorption of a multivalently bound protein from an alkyl-Sepharose at

the optimal residue density is best obtained by a procedure that decreases the affinity and capacity of the gel by one or two orders of magnitude. In general, this is most easily achieved by increasing salt gradients in salting-in chromatography[2] and by decreasing salt gradients when salting-out chromatography is employed. The type of salt should be chosen from the Hofmeister series[1].

Temperature gradients alone are not likely candidates for an efficient elution procedure. As can be concluded from Fig. 1, a temperature change of *ca.* 30° leads to a relatively small change in affinity (compare the $K_{0.5}$ values) and to only a 30–40% change in gel capacity. Therefore, the above conditions for efficient elution are not fulfilled.

3.5. Nomenclature

A problem in all newly emerging and rapidly developing fields is the definition of terms. In Fig. 2 an attempt has been made to enumerate in a systematic manner some of the terms so far suggested for the different types of adsorption chromatography on substituted gels. The term *univalent interaction chromatography* is taken to classify adsorption described by Langmuir-type isotherms which indicate single, independent binding sites. Multiple contacts occurring in single, non-overlapping binding units may, however, not be differentiated by such isotherms. It appears that

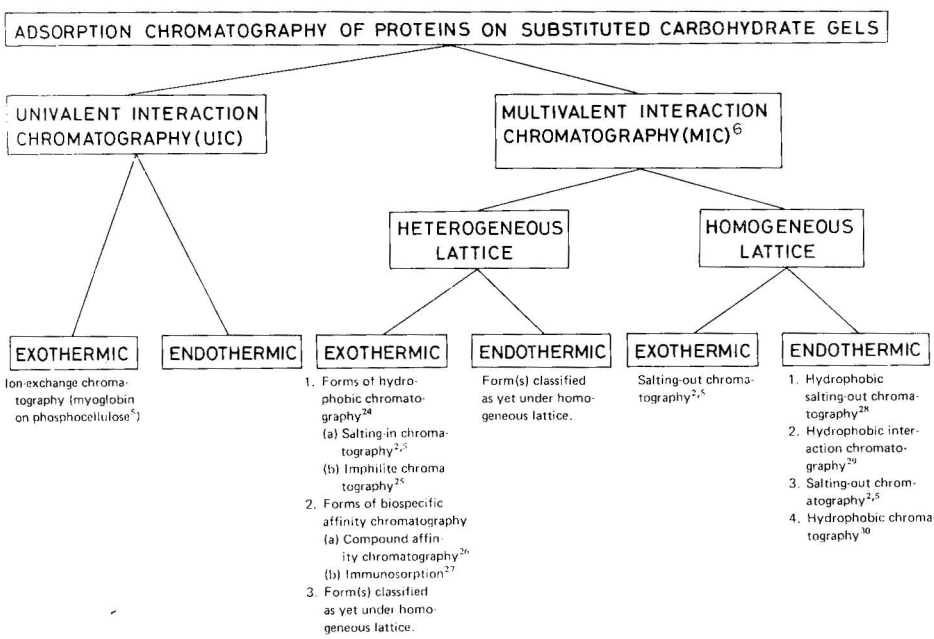

Fig. 2. Tentative classification of the nomenclature frequently employed for the various types of adsorption chromatography on carbohydrate gels. Heterogeneity and homogeneity are defined here on a qualitative basis, *e.g.*, a heterogeneous lattice may contain effective binding sites of both ionic and hydrophobic character. On a homogeneous lattice it is assumed that the effective binding sites are qualitatively homogeneous, *e.g.*, exclusively ionic or hydrophobic in nature. For further details, see the text and the references cited.

virtually all forms of chromatography defined by the listed terms employing substituted agarose gels are based on multivalent matrix-ligand interactions. Therefore, the general term *multivalent interaction chromatography* has been proposed[6]. The subgroup to which the listed forms belong has in most instances still to be determined by thermodynamic and binding experimental methods.

In the group, exothermic multivalent interaction chromatography on an effective, qualitatively heterogeneous lattice of binding sites, those chromatographic types which are performed at initially low ionic strength are listed and to which "detergent chromatography"[32] may be added. All forms of chromatography at high ionic strength ($\mu > 1.0$) to which "phosphate-induced chromatography"[33] may be added are tentatively listed under multivalent interactions on an effective, qualitatively homogeneous lattice under the assumption that interactions depending primarily on water structure (*e.g.*, surface tension effects and hydrophobic interactions) are the basis for adsorption.

An unambiguous classification of ion-exchange chromatography (*e.g.*, myoglobin on phosphocellulose[5]) and salting-in chromatography (*e.g.*, phosphorylase *b* on methyl- and butyl-Sepharose, at low ionic strength[5]) is possible. In exothermic and endothermic salting-out chromatography (phosphorylase *b* on methyl- and butyl-Sepharose, respectively, at high ionic strength[5]), an effective, homogeneous lattice is proposed as ionic interactions are improbable. However, especially in exothermic salting-out chromatography, the exact adsorption mechanism has still to be elucidated.

From the large number of different terms often employed for identical or similar procedures, it appears that the time is now ripe for the IUPAC Nomenclature Commission to decide on clear definitions.

4. ACKNOWLEDGEMENT

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5. SUMMARY

Cyanogen bromide-activated Sepharose 4B substituted with α-aminoalkanes (1–4 carbon atoms) has been shown to adsorb proteins by multivalent interactions (multivalent interaction chromatography) at low and at high salt concentrations (salting-in and salting-out chromatography, respectively). Equilibrium binding studies and desorption kinetics indicate that the affinity of alkyl-Sepharoses decreases as a function of fractional saturation (negative cooperativity). Temperature-induced changes in gel capacity appear to be linked to the minimum valence of adsorption.

In spite of these very complex ligand–matrix interactions, such gels can easily be optimized for a 10–20-fold chromatographic purification of proteins from crude extracts. The degree of substitution (alkyl-residue density) at the chromatographic optimum corresponds closely to the alkyl-residue density of half-maximal saturation, $(K_{0.5})^{-1}$. This value can be determined from a Hill plot of the binding capacity of the gel *versus* the alkyl-residue density. The same plot simultaneously yields the minimum number of binding sites involved in the adsorption of the protein.

An attempt is made to classify the confusing nomenclature employed in adsorption chromatography in terms of univalent and multivalent interactions.

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FRACTIONATION OF MEMBRANE PROTEINS BY HYDROPHOBIC INTERACTION CHROMATOGRAPHY AND BY CHROMATOGRAPHY ON AGAROSE EQUILIBRATED WITH A WATER–ALCOHOL MIXTURE OF LOW OR HIGH pH

STELLAN HJERTÉN

Institute of Biochemistry, University of Uppsala, Biomedical Centre, Box 576, S-751 23 Uppsala (Sweden)

CONTENTS

1. INTRODUCTION

In other papers at this Symposium it has been shown that hydrophobic interaction chromatography can be used for the purification of water-soluble proteins, and that low-molecular-weight substances can be separated from proteins on neutral amphiphilic (amphipathic) beds. It is perhaps not so well known that hydrophobic interaction chromatography can also be used for the purification of particles, for instance viruses and even whole cells[1]. In fact, in collaboration with Prof. Torkel Wadström and Dr. Cyril Smith, we are using this chromatographic technique to classify bacteria. However, the applications considered in this paper deal with proteins from biological membranes.

2. COMMENTS ON THE SOLUBILIZATION OF MEMBRANE PROTEINS

The proteins in biological membranes form complexes with each other and with the constituents of the lipid bilayer. The interactions between the components in these complexes involve electrostatic, hydrophobic, Van der Waals and hydrogen bonds, all of which must be broken before complete solubilization of the membrane can take place. Accordingly, membrane proteins are not soluble in conventional buffer systems. Any purification study must therefore start with a search for a suitable solubilizing medium.

If irreversible denaturation of the proteins can be tolerated, the problem is very simple in most instances, as one can then choose buffers containing sodium dodecyl sulphate (SDS) (which, however, has been reported to be reversibly denaturing for

some enzymes)[2,3]. The problem is considerably more difficult if one must also preserve biological activity, for instance an enzyme activity. In such instances one must choose bile salts, for instance sodium deoxycholate (DOC), or neutral detergents such as Tween 20 or Triton X-100. Of these, Triton X-100 has been most widely used. However, its high UV absorption is a great disadvantage in fractionation studies. Therefore, we often use the non-UV-absorbing detergent G 3707 (Atlas Chemicals, Everberg, Belgium), which seems to be as efficient as Triton X-100[4]. As none of these detergents is as efficient as SDS, it is often necessary to supplement the medium with certain additives in order to suppress certain interactions. In such studies it is important to bear in mind that the addition of salt decreases the electrostatic but increases the hydrophobic interaction and *vice versa*. It is also known that different salts affect the hydrophobic bond to different extents (experiments have shown that the salts can be arranged in a Hofmeister series as to their effect on this bond[5–7]). For these reasons, it is often only at a certain concentration that a given salt (buffer) effects solubilization.

From these considerations, it is evident that one should investigate the solubilizing effect of a series of different additives at different concentrations and at different pHs. In this connection, it should be stressed that a clear, non-opalescent solution of membrane proteins is not necessarily free of large complexes. If the protein of interest forms part of such a complex it can, of course, never be isolated in a pure form. It can be difficult to decide whether such complexes exist, as they often contain lipids and therefore do not easily sediment in the ultracentrifuge even at high g values.

3. GENERAL ASPECTS OF THE PURIFICATION OF MEMBRANE PROTEINS

Even if we have managed to get the protein into solution in a free form, many problems will arise in connection with purification studies, *e.g.*,

(1) Most proteins solubilized in SDS have the same surface charge density (ζ-potential). They therefore cannot be separated by carrier-free electrophoresis or electrophoresis in a non-sieving supporting medium such as agarose. The same applies to a buffer containing DOC although to a lesser extent.

(2) In the presence of neutral detergents, the proteins will be less charged and therefore often migrate relatively slowly in an electrical field.

(3) The micelles of most detergents have relatively high molecular weights (in the range 20,000–80,000) and it is therefore virtually impossible to remove them by dialysis. An exception is the bile salts, with micellar molecular weights around 3000. They also differ from some other detergents in that they have a relatively high critical micellar concentration (CMC) of about 5 mM in water, which also contributes to a comparatively rapid removal by dialysis[8,9]. The recently introduced detergent octyl glycoside is extremely easy to remove by dialysis as it has a very high CMC (about 25 mM).

(4) As the detergents are bound to the membrane proteins in large amounts (often 1.4 g of SDS per gram of protein[10]), the relative differences in molecular weights between complexes of protein and detergent are often too small to permit a fractionation by chromatographic molecular sieving. Also in this instance the bile salts are preferable to other detergents owing to the low molecular weights of their micelles (bile salts and neutral detergents seem to be bound to proteins in the form of micelles).

(5) It is well known that different polymers are incompatible. Solutions of, for instance, non-crosslinked dextran and polyacrylamide will precipitate proteins. It is therefore not surprising that many hydrophobic membrane proteins precipitate or form complexes with other proteins when applied to chromatographic columns of crosslinked dextran or polyacrylamide. Also, the aggregates that often are visible at the top of analytical polyacrylamide gel electrophoresis columns can have their origin in similar incompatibilities. In some instances it is therefore preferable to utilize methods that do not require the presence of supporting media.

From the above considerations, it is evident that conventional fractionation methods have an inherent weakness when used for the separation of membrane proteins. We have therefore felt the need for novel techniques to isolate these water-insoluble biopolymers. Hydrophobic interaction chromatography was developed with this in mind, but we soon realized that this method was also useful for common water-soluble proteins. When employed with membrane proteins one should remember that the detergents added to the medium to keep the membrane proteins in solution interact with the non-polar ligands of the bed material (in addition to the hydrophobic "patches" on the surface of the proteins), thereby decreasing the adsorption of the proteins to the bed. If excessively high detergent concentrations are used no adsorption will take place unless the hydrophobicity of the ligands is increased. When all ligands have reacted with the detergent molecules (in the free form or in the form of micelles), one has created a new adsorbent with properties different from those of the original bed.

4. APPLICATIONS

The general aspects and comments given above on the solubilization of membrane proteins with the aid of detergents should be borne in mind in the following discussion of applications. Relatively few experiments have been reported in which hydrophobic interaction chromatography has been used for the fractionation of proteins from biological membranes. Weiss and Bücher[11] employed a cation exchanger with lipophilic ligands to separate mitochondrial membrane proteins. Hjertén[12] purified a protein (called T_b) from the membrane of *Acholeplasma laidlawii* by chromatography on Sepharose to which phenylethylamine had been attached by the cyanogen bromide method[13]. As the nitrogen in the amine partly retains its positive charge after the coupling, the separations obtained on such a bed are based upon both hydrophobic and electrostatic interactions. The same is true for the bed material used by Simmonds and Yon[14] for the fractionation of proteins from erythrocyte membranes.

However, upon coupling alcohols to Sepharose by the glycidyl ether method, no charges are introduced[1]. These neutral amphiphilic columns, the advantages of which have recently been pointed out[15], have also been used for the purification of membrane proteins from *Acholeplasma laidlawii*[15,16]. Another example is given in Fig. 1, which shows the chromatographic behaviour of erythrocyte membrane proteins on dodecyl-Sepharose in the presence of sodium dodecyl sulphate. Desorption was achieved by first decreasing the buffer concentration and finally by increasing the SDS concentration. The protein distribution in the effluent was determined by absorption measurements at 280 nm and the phospholipid distribution was measured by phos-

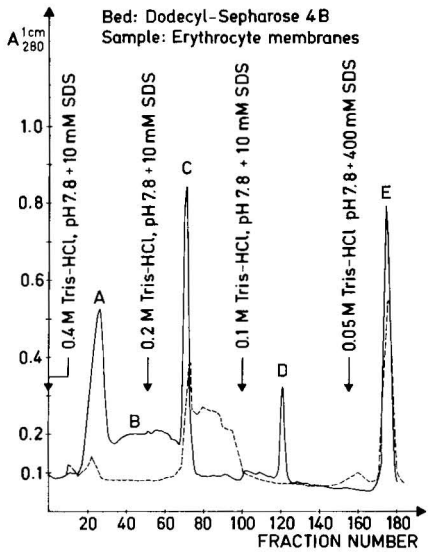

Fig. 1. Hydrophobic interaction chromatography of human erythrocyte membrane proteins on dodecyl-Sepharose. Sample amount: 10 ml (45 mg) in 0.4 *M* Tris–HCl, pH 7.8, +20 m*M* SDS. Column dimensions: 28 × 1.4 cm. Flow-rate: 13 ml/h. Fraction volume: 2.2 ml. Temperature: 21°. The broken curve corresponds to phosphate measurements.

phate determinations. The materials corresponding to the different peaks were analysed by polyacrylamide gel electrophoresis in the presence of SDS. As is evident from Fig. 2, this analysis revealed that peak A corresponded to highly purified glycoproteins (PAS-1 and PAS-2, the former being a dimer of the latter[4,17]). A similar result

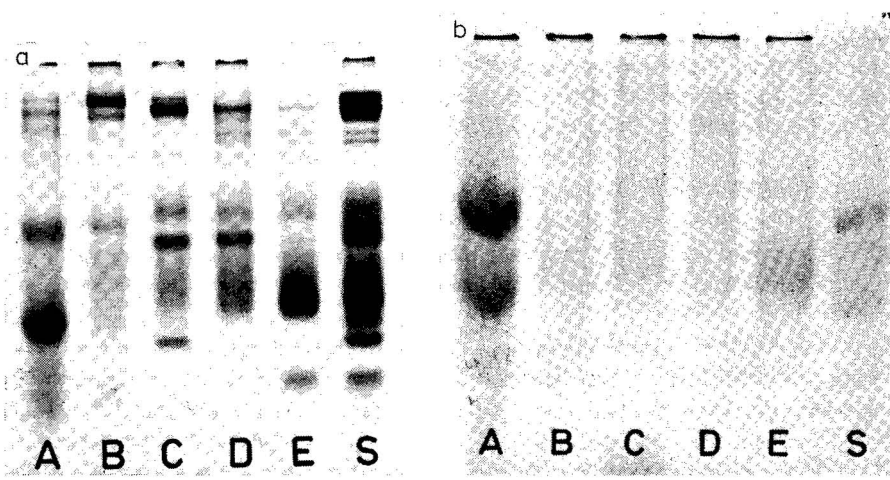

Fig. 2. Analysis of the chromatogram in Fig. 1 by SDS electrophoresis. Gel: polyacrylamide of the composition T = 6%, C = 3% (these notations are defined in ref. 18). Buffer: 0.05 *M* glycine–NaOH, pH 9.8, +0.02 *M* SDS. (a) Staining for proteins with Coomassie Brilliant Blue; (b) PAS staining for glycoproteins. The sample denoted by S corresponds to the unfractionated starting material, which was somewhat degraded, probably by proteolysis.

has been reported by Simmonds and Yon[14]. Fig. 2 also indicates that the other fractions contained non-glycoproteins of different degrees of purity. In the paper on the behaviour of proteins of Sepharose at low pH, it was mentioned that membrane proteins are strongly adsorbed to this adsorbent equilibrated with butanol–acetic acid–water[19]. This observation prompted us to try to devise a new separation method. Even though it still is in the developmental stage, it has already been used for a practical fractionation problem, namely, the isolation of the glycoproteins from erythrocyte membranes (see Fig. 3). Fig. 4 shows that all of the glycoproteins are collected in fraction A, which contained no other proteins.

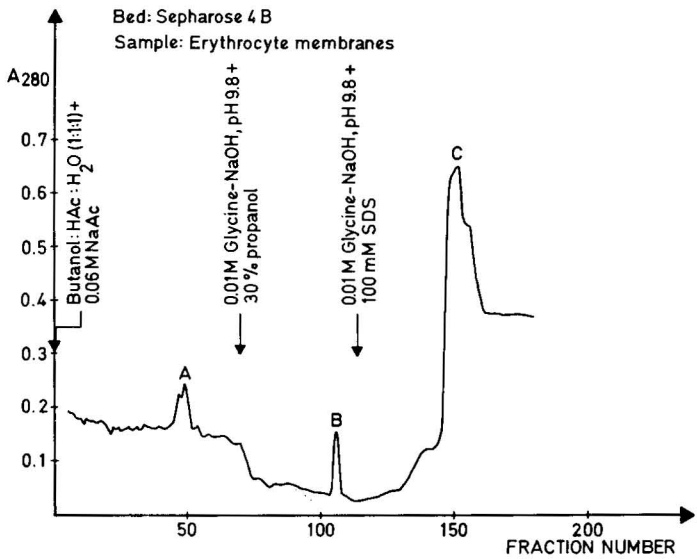

Fig. 3. Chromatography of human erythrocyte membrane proteins on Sepharose 4 B equilibrated with butanol–acetic acid–water (1:1:1). Sample amount: 2 ml (18 mg) solubilized in 4 ml of butanol–acetic acid–water (1:1:1) containing 0.06 *M* sodium acetate. Column dimensions: 70 × 2 cm. Temperature: 21°. Flow-rate: 6 ml/h. Fraction volume: 6 ml. The column was equilibrated with the same medium as was used to solubilize the sample.

5. DISCUSSION

The above experiments have shown that membrane proteins can be separated by hydrophobic interaction chromatography. The resolving power is, however, much lower than that of polyacrylamide gel electrophoresis, as shown by comparison of Figs. 1 and 2a. Similar differences in resolution between electrophoresis and chromatography are obtained when membrane proteins are chromatographed on gel beads of dextran, polyacrylamide and agarose. Can these differences originate partly from the tendency of (membrane) proteins to form complexes and to precipitate in the presence of polymers (see point 5 above)? If such is the case, there should be a greater tendency for aggregation in chromatography on gel beads than in electrophoresis in homogeneous, coherent gels. This might well be so, as proteins migrating in the pores of a polyacrylamide gel do not come into contact with each other to the same extent

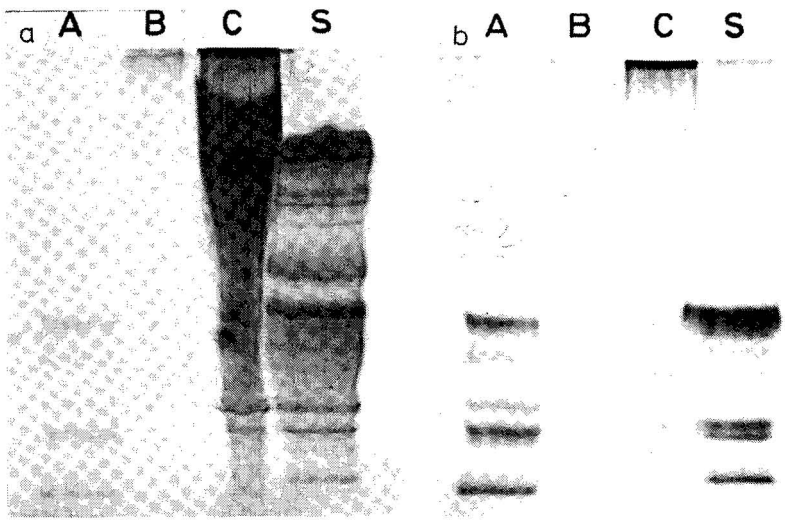

Fig. 4. Analysis of the chromatogram shown in Fig. 3 by pore-gradient electrophoresis in SDS. The gradient gels, obtained from Pharmacia Fine Chemicals (Uppsala, Sweden), had the notation PA A4/30. Buffer: 0.05 *M* glycine–NaOH, pH 9.8, +5 m*M* SDS. (a) Staining for protein with Coomassie Brilliant Blue; (b) PAS staining for glycoprotein. The sample denoted by S corresponds to the unfractionated starting material.

as they do in chromatography (contact is, of course, a prerequisite for the formation of aggregates*). A similar hypothesis can also explain why proteins precipitate much more easily when they are submitted to isoelectric focusing in a sucrose gradient than in a polyacrylamide gel.

It is a characteristic of several chromatographic methods, including hydrophobic interaction chromatography, that most often only one peak is obtained with each buffer used for desorption (see Fig. 1). However, when Tween 20, a neutral detergent, is used several peaks usually appear in each elution step. (see Fig. 5 in ref. 15 and Fig. 4 in ref. 16). The reason for this has not been explored, but the effect should be borne in mind as this type of elution is highly desirable.

From this paper, it is evident that the presence of detergents causes several difficulties in connection with the purification of membrane proteins. We have, however, made the very important observation that many membrane proteins are soluble in conventional buffers in the absence of detergents, provided that the proteins are extensively purified (see the paper by Moore[21], who has made a similar observation). This means that in the last stages of a purification scheme one need not always use detergents, which facilitates the purification. It should also be pointed out that many membrane proteins are soluble in the absence of detergents after carboxylation of the amino groups, for instance with dimethylmaleic acid[20]. By changing the pH the protein can easily be decarboxylated. Detergents can also be avoided by solubilizing the membranes in butanol–acetic acid (morpholine)–water (Fig. 3).

* Point 5 above should also be considered in a discussion of aggregate formation.

6. ACKNOWLEDGEMENTS

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7. SUMMARY

Some general aspects of the solubilization and purification of non-water-soluble membrane proteins are given. Hydrophobic interaction chromatography can be used for the fractionation of such proteins provided that the detergent concentration and the hydrophobicity of the ligands are properly selected.

Columns of agarose equilibrated with butanol (or propanol)–acetic acid (or morpholine)–water have also been used for the fractionation of membrane proteins, but media suitable for desorption of all proteins have not yet been found.

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HEXOKINASES AND MYOSIN: A PROBLEM OF ISOENZYME SEPARATION

IAN P. TRAYER

Department of Biochemistry, University of Birmingham, P.O. Box 363, Birmingham B15 2TT (Great Britain)

CONTENTS

1. INTRODUCTION

From its inception in 1968[1], affinity chromatography for protein purification has utilized immobilized substrates or substrate analogues primarily for the adsorption and elution of a single enzyme. "Group specific" or "general ligand" affinity chromatography, where a ligand capable of interacting with a wide variety of enzymes is immobilized, was a more recent development of this procedure and has been used extensively for the separation of nicotinamide nucleotide-dependent dehydrogenases on columns containing covalently-bound AMP or $NAD(P)^+$ derivatives[2,3]. Further developments of the technique have made possible a study of a variety of protein–ligand interactions, *e.g.* an investigation of enzyme mechanistic processes[3,4], separation of the lactate dehydrogenase isoenzymes[5] and the study of complex formation between enzymes and other ligands[6]. Indeed, it has been possible to use this technique to obtain accurate values for the binding constants between the protein and the immobilized ligand and eluting ligand[7–9].

By definition, affinity chromatography relies on the existence of a simple equilibrium between the immobilized and free interacting components. Thus, it is advisable to adapt any aspect of the free solution kinetic behaviour of the various enzyme–ligand interactions to the chromatography system under study. In this way, the specificity of both the adsorption and desorption phases can often be greatly increased with concomitant benefit to the experiment. The purpose of this report is to illustrate how this relationship between the free solution and chromatographic behaviour has been developed in our studies on the purification of the mammalian hexokinase isoenzymes and the fractionation of vertebrate skeletal muscle myosin isoenzymes. In the former case, the problem was essentially one of protein purification whereas although myosin isoenzymes have been suspected of existing for the last decade[10] it is only very recently that this has been established unequivocally[11].

2. STUDIES ON THE HEXOKINASE ISOENZYMES

There are four hexokinase isoenzymes found in mammals, numbered I to IV in order of increasing acidity on electrophoresis, whose distribution is largely tissue-specific; type I is found principally in the brain, heart and kidney, type II in skeletal muscle and adipose tissue, type III is found in all tissues in very small amounts but principally in the liver and spleen, whereas type IV, also known as glucokinase, is restricted to the liver and is the main glucose phosphorylating enzyme of that tissue. Although the glucose phosphorylation step is a prime candidate for regulation in metabolism, lack of efficient purification procedures for these enzymes has hindered any in depth investigations into their physiological control and mode of action. Until recently the only isoenzyme to be purified to homogeneity was the type I from brain[12,13] and pig heart[14]. The selective extraction of the mitochondria, to which this isoenzyme is bound, simplified the purification process but the other three isoenzymes could not be obtained so easily. However, Holroyde and Trayer[15] and Holroyde *et al.*[16,17] succeeded in purifying glucokinase to homogeneity using, in conjunction with ion-exchange chromatography, affinity chromatography on a Sepharose-bound glucosamine derivative [N-(6-aminohexanoyl)-2-amino-2-deoxy-D-glucopyranose].

Initial experiments with this matrix[18] suggested that the glucokinase bound selectively and reversibly but that the low K_m hexokinase activity (presumed to be a mixture of hexokinase types I, II and III) in liver extracts and yeast hexokinase were unretarded. At first this was a puzzling observation since it is well known that both glucosamine and N-acetylglucosamine are good inhibitors of all four isoenzymes[19]. Eventually, it was found that hexokinase type II from skeletal muscle could be purified to homogeneity on this matrix[20]. This prompted a more thorough investigation of the reactions between the hexokinases and the glucosamine derivatives. Various glucosamine derivatives were synthesized giving a series of compounds in which the length of the polymethylene chain between the amide linkage to glucosamine and the primary amino group for attachment to the Sepharose matrix was varied. These glucosamine derivatives [N-aminopropionyl-(C3), N-aminobutyryl-(C4), N-aminohexanoyl-(C6) and N-aminooctanoyl-(C8)] had the effect of altering the length of the spacer molecule attaching the glucosamine to the Sepharose.

All of these N-aminoacyl glucosamine derivatives in free solution proved to be competitive inhibitors with respect to glucose of the rat and yeast hexokinases but with differing K_i values[21]. K_i can be taken as a direct measure of the dissociation constant in these systems. In fact, a direct correlation was found to exist between these kinetic data and the effectiveness of a particular derivative as an affinity chromatographic medium when immobilized to Sepharose. Thus the only really effective inhibitor for rat kidney hexokinase type I was the C-8 glucosamine derivative (K_i = 1.3 mM) and only the Sepharose conjugate of this derivative proved a suitable affinity matrix. This isoenzyme was probably responsible for our initial observations of low K_m hexokinase activity appearing in the wash-through fraction when liver extracts were applied directly to the Sepharose-C6 glucosamine matrix[18]. Subsequent chromatography experiments showed that providing the K_i for the ligand was $\leqslant$2 mM then a suitable affinity matrix could be constructed. For example, hexokinase type II could be chromatographed successfully on any of the immobilized derivatives since in free solution its K_i for all four glucosamine derivatives was less than 2 mM. Similarly,

glucokinase and hexokinase type III could be adsorbed most effectively to the immobilized C-6 and C-8 glucosamine derivatives. Furthermore, in situations where the K_i of two isoenzymes for a particular ligand differed (but were both less than 2 mM) these enzymes could be separated by either adjusting the ligand concentration attached to the gel or employing a glucose gradient for elution. Hexokinase type II ($K_i = 0.4$ mM) and glucokinase (0.75 mM) could be separated on a Sepharose-N-aminohexanoylglucosamine conjugate in this way. It should be pointed out that none of the hexokinases showed any interaction with Sepharose derivatives formed by linking either glucosamine or the various amino acid spacer molecules directly to the support. Although these initial experiments demonstrating the relationship between the free solution behaviour and the subsequent chromatographic behaviour were carried out on a small scale, it has been possible to handle large quantities of tissue extracts in this way. Consequently, in conjunction with conventional methods, it was possible to purify to homogeneity hexokinase type I from 500 g of rat kidneys, and hexokinase types II and III from the livers of 50 rats on Sepharose-N-(8-amino-octanoyl)glucosamine matrices.

These experiments illustrate the usefulness of adapting the kinetic behaviour to the affinity adsorbent operation. Thus, by varying either the length of the spacer molecule and/or the concentration of ligand attached to the gel[16] a glucosamine affinity matrix can be designed specifically for the purification of each hexokinase isoenzyme. These data also suggest that, at least with this hexokinase system, the spacer molecule appears to be making a specific contribution to the enzyme–immobilized ligand interaction. This was very apparent during our comparative investigations with yeast hexokinase. Our initial experiments indicated that this hexokinase would not bind to our C6-glucosamine matrix. Subsequent kinetic studies revealed that as the length of the spacer molecule was increased up to six C atoms, the K_i had increased to about 24 mM. It was a surprise, however, that when an additional two methylene groups were added, *i.e.* the C8-glucosamine, the K_i decreased to 1 mM and an efficient chromatographic adsorbent has been prepared from this derivative.

There have been reports in the literature that yeast hexokinase will not bind efficiently to an ADP-affinity adsorbent[22]. Studies on the kinetics of this enzyme (for review see ref. 23) show that although the enzyme exhibits a random ordered addition of substrates at high $Mg \cdot ATP^{2-}$ concentrations, a mechanism by which glucose binds before $Mg \cdot ATP^{2-}$ is the preferred pathway. The affinity chromatographic behaviour of the enzyme substantiates this behaviour. If yeast hexokinase is applied to an N^6-(6-aminohexyl)-ADP-Sepharose matrix (N^6-ADP) in the absence of glucose the enzyme is partially retarded but efficient binding to the column is only observed when glucose is included in the application buffer.

3. STUDIES ON THE MYOSIN ISOENZYMES

Myosin from the fast twitch muscles of rabbit is hexameric and comprises two polypeptide chains of molecular weight approximately 200,000 (heavy chains, HC) and four moles (per mole of myosin) of light chains: two moles of identical phosphorylatable polypeptides of molecular weight 18,000 (the P-LC) and two moles (total) of two further polypeptides of molecular weight 22,000 and 16,000 (the so-called alkali light chains, A1 and A2 respectively, named after their method of removal

from the native myosin). In overall design the myosin molecule is rod-shaped and divided at one end into two globular head regions (for review see ref. 24). The HC run throughout its length, whereas the light chains are associated exclusively with the head regions; the P-LC are possibly located near the junction between the rod and head regions[25,26] and the alkali light chains reside within the globular head regions, which possess both the actin-binding and ATPase activities of the myosin.

We have been using affinity chromatography techniques in our studies on the structure and function of myosin to answer two problems: the role of the light chains in the interactions of this protein with actin and ATP and whether or not myosin from vertebrate skeletal muscle exists as isoenzymes. Myosin from heart muscle, from many non-vertebrate muscles and from non-muscle sources invariably contains only 2 moles each of two different light chains per 2 moles of HC and with these proteins the subunit structure of the hexameric molecule is easy to envisage. Densiometric and radiochemical studies on rabbit fast twitch muscles have shown that the A1 and A2 light chains exist in a molar ratio of 1.35:0.65 respectively[27,28]. The presence of different heavy chain populations in myosin isolated from these species is also indicated by the observation of amino acid substitutions in certain peptide sequences[29]. Thus, the existence of myosin isoenzymes has frequently been invoked to explain these differences, but until recently[11] these had not been separated. With respect to light chain composition, each myosin head contains one P-LC and three myosin isoenzymes are suggested based upon the alkali light chain distribution: the symmetrical homodimers A1/A1 and A2/A2 and an A1/A2 heterodimer. Since myosins isolated from many vertebrate skeletal muscles contain a P-LC and two other electrophoretically distinct light chains, the existence of isoenzymes is likely to be a widespread phenomenon. The difficulties in separating any isoenzymes is compounded by the fact that myosin is insoluble at low ionic strength and so cannot be easily subjected to normal electrophoretic techniques. Fragments of myosin that are soluble at low ionic strength and that contain the biological activities of the parent molecule have been prepared for a number of years by proteolysis with different proteases. Much of the work on the functioning of myosin has thus been carried out using the water-soluble proteolytic fragments, heavy meromyosin (HMM), which contains both heads and half the rod portion of the molecule, and subfragment-1 (S-1), which is a preparation of the separated, single globular heads. Until recently the large number and variety of polypeptide components found on SDS-gel electrophoresis of these digests made it difficult to identify their origin unambiguously. By controlled digestion with chymotrypsin, however, Weeds and his co-workers[26,30] were able to obtain S-1 preparations that contained a single HC species (of molecular weight approximately 90,000) and the alkali light chains (with total loss of the P-LC) and HMM preparations that also contained a homogeneous HC species (of molecular weight about 140,000) and a full complement of light chains.

Chymotryptic S-1 from rabbit skeletal muscle myosin could be fractionated on columns of either Sepharose-bound G-actin[31] or F-actin (stabilized by glutaraldehyde cross-linking) into species containing only the A1 light chain [S-1 (A1)] or the A2 light chain [S-1 (A2)] by elution with gradients of ATP, ADP or PP_i (either as their free acids or Mg salts) or even KCl (ref. 32). In all cases the S-1 (A1) species required a higher concentration of nucleotide to dissociate it from the immobilized actin than the S-1 (A2) species. This observation agrees with the free solution kinetic analyses where

the apparent K_m for actin of the S-1 (A1) is less than that for the S-1 (A2)[30,33]. These results are not unique to S-1 populations isolated from the myosin of fast-twitch rabbit muscle. Corresponding experiments with S-1 populations isolated from myosin from rabbit slow-twitch muscle and chicken breast muscle also revealed two light chain species of S-1 with distinct binding properties[32]. Again the S-1 species containing the larger light chain showed the greater affinity for actin, but both species bound more tightly to the actin matrix, as judged by the nucleotide concentration required for elution, than the corresponding S-1's from the rabbit fast-twitch muscle. Indeed, a careful comparison of the elution profiles of these S-1's and bovine cardiac muscle S-1 (whose myosin contains only one type of alkali light chain) revealed a spectrum of actin-binding affinities which are in accord with their apparent K_m values for actin[33]. Furthermore, S-1 species isolated from slower-twitch muscle myosins bind to the actin matrices more tightly than those from fast-twitch muscles.

The experiments described here do not distinguish between the relative roles played by the heavy and light chains in actin binding. However, a role for the alkali light chains in determining the strength of binding to actin is indicated when these results are taken in conjunction with the observations of Wagner and Weeds[34]. The authors showed that the differences observed in the actin-activated ATPase activities of isolated native and hybridized rabbit S-1 populations were solely due to the particular alkali light chain present. It is noteworthy that the larger A1 light chains of myosin from chicken breast and rabbit white skeletal muscle and the cardiac light chain all contain proline and alanine-rich N-terminal regions[35,36] and it is tempting to speculate that these segments are responsible for the enhanced actin binding, especially of the S-1 (A1) types over the S-1 (A2) species from the same muscle.

S-1 populations from rabbit fast-twitch and slow-twitch muscle and from chicken breast muscle could also be reversibly bound to columns of Sepharose-bound ADP derivatives [N^6-ADP and 8-(6-aminohexyl)amino-ADP; ref. 37]. In each case, the two populations of S-1 could be fractionated when the columns were developed with ADP^{3-} or ATP^{4-} but not when either their Mg^{2+} or Ca^{2+} salts were used[38]. This is very interesting since no differences in either the Mg or Ca·ATPase activities of the separated S-1 species could be found.

Experiments with S-1 populations do not, however, help to decide about the existence of myosin isoenzymes since the A1- and A2-containing species could have originated from the same or separate myosin molecules. Thus, similar experiments to the above were carried out with chymotryptic HMM prepared from myosins isolated from both rabbit fast-twitch and chicken breast muscles. These HMM preparations could be fractionated into their homodimers on columns of matrix-bound actin, ADP and pyrophosphate using gradient elution techniques but this was much more difficult than fractionation of the S-1 populations. It was necessary to employ long thin columns (up to 30 × 0.9 cm) with long gradients to achieve the best separations and even so these were incomplete. The protein eluting at the lowest nucleotide concentration contained only the A2/A2 homodimer whereas that eluting at the highest nucleotide concentration contained only the A1/A1 homodimer. In between, HMM containing all three light chains was found. Similar fractionations were obtained from each affinity matrix using a variety of eluants: ATP, ADP, PP_i (free and as their Mg salts) and KCl. These studies clearly demonstrate the existence of homodimers but do not entirely eliminate the possibility of heterodimers being present. As yet we have been

unable to obtain two clearly separated peaks of homodimers. However, when the central portion of such a profile, containing all three light chains, is taken and re-applied to a second affinity column, then the material re-fractionated as above. A third re-run of the central portion again fractionated as well as the first application. These data suggest that homodimers predominate, at least, and any heterodimers must be present in only very small amounts.

Thus, careful affinity chromatographic studies have helped to elucidate the basic structure of the myosin molecule and the probable role of the alkali light chains. Each myosin molecule contains two moles of identical (alkali) light chains situated within the head region, which control the strength of binding of myosin to actin, and two moles of metal-binding (phosphorylatable in vertebrates) light chains[25], possibly arranged at the head–tail junction[25,26], which play a role in the regulation of the actomyosin ATPase. A regulatory role for these light chains in myosins from molluscan[39] and smooth[40] muscle systems and in some non-muscle myosins[41] is well-documented. Their exact role in vertebrate skeletal muscle myosins is as yet unknown although their phosphorylation and dephosphorylation by a specific kinase and phosphatase is established[42,43]. It seems that only vertebrate skeletal muscle myosin populations possess two types of actin-binding (alkali) light chains, suggesting that isoenzymes are invoked in cases where both maintained muscle tone and variation in contractile speed are required, and that this is achieved by the presence of myosin species which differ in their affinity for actin and therefore in their ability to generate force.

4. ACKNOWLEDGEMENTS

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5. SUMMARY

A direct correlation exists between the ability of various N-aminoacyl-glucosamine derivatives (propionyl, butyryl, hexanoyl and octanoyl) to inhibit the four mammalian hexokinase isoenzymes and yeast hexokinase in free solution and their effectiveness as affinity chromatographic media for these enzymes when immobilized to Sepharose. Thus, by using Sepharose conjugates with different spacer molecules and/or adjusting the final ligand concentration attached to the gel, glucosamine affinity matrices have been designed to purify specifically each isoenzyme on a large scale. Chromatography of vertebrate skeletal myosin on columns of immobilized actin or ADP has shown that these myosin preparations contain mixtures of symmetrical homodimer isoenzymes with respect to the light chains, *i.e.* each head of the myosin molecule is identical and contains two different light chains. Functionally these homodimers differ in their ability to bind to actin.

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NEW APPARATUS FOR ISOELECTRIC FOCUSSING

A. J. P. MARTIN and F. HAMPSON
School of Molecular Sciences, University of Sussex, Brighton BN1 9QJ (Great Britain)

CONTENTS

1. INTRODUCTION

The method of isoelectric focussing would be an extremely elegant and useful first step in the separation of proteins. The property being used for the separation is well defined and can readily be determined on small samples using analytical apparatus. This would enable one to get rid of the vast majority of contaminating molecules. So far, however, no apparatus has been available in which large quantities of material can be treated. The analytical methods devised by Svensson[1,2] and Vesterberg and Svensson[3] used a separating medium of carrier ampholytes, consisting of amphoteric buffers with a wide spectrum of isoelectric points, which are conducting when isoelectric. This medium is unfortunately too expensive for use on a large scale, and at pH values widely different from neutrality, affords only a limited time for separation because of its drift towards the electrodes. Therefore, our approach has been to devise a system using only simple buffer solutions. Early work on isoelectric focussing in simple buffers has been reviewed by Kolin[4].

Another condition for large-scale work is efficient removal of heat from the electrolytes. We have chosen to use a series of compartments separated by diaphragms which individually possess isoelectric points, so chosen that the compartments can be maintained at a graded series of pHs. The contents of each compartment are cooled by being pumped through an external electrically insulated heat exchanger. The distance between successive membranes is reduced to the minimum that will allow an adequate flow of electrolyte and thereby as little heat as possible is generated. The extensive early work on compartment apparatus using non isoelectric separators has been reviewed by Svensson[5], who has also suggested the use of amphoteric membranes but not in a situation where they would be isoelectric[1].

With simple buffers it is only possible to maintain stable pHs in such an ap-

paratus with a single pair of electrodes, if one ensures that the transport number of the anions and cations of the buffer is constant throughout the apparatus. This can only be attained by using monovalent ions of each charge, one or both of which may be buffering. Means also have to be provided to maintain the pH of the end compartments at a stable level. This can be done in two ways. (1) Buffer solution can run through the end compartments to waste at a sufficient rate, or (2) the buffer ions can be returned by pumping liquids between the two electrode compartments at the correct rate. The latter method can only be employed if the material to be separated contains no ions which can migrate outside the limit of the pH steps between the end amphoteric membranes. The use of monovalent buffering ions limits the range of pH to about 1.5 units in the case where one ion only is buffering, and to about 3.5 units if both ions are buffers and are chosen with a suitable interval between their pK values. This is not, in fact, a very severe limitation if the aim is to isolate from a mixture a single species with a given isoelectric point. The pH of the buffer can be chosen as optimal for this particular separation and other substances can be discarded, without the necessity of separating them from each other. Of course the purpose of an analytical apparatus is to separate the complete spectrum, but for preparative work the apparatus need only cover a pH range with that of the wanted substance near its centre. By complicating the apparatus and having electrodes for each compartment, it is possible to use multivalent buffer ions with a very much wider buffering range but whose transport number depends on the degree of ionisation.

If the mixture to be purified contains multivalent ions then its rate of addition must be limited to a value which does not cause such a large disturbance of pH that the wanted substance is lost from the apparatus. The pH values should be readjusted before counter-flow is begun.

2. THE ELECTROPHORESIS APPARATUS

Our early work was done with thick agarose gels which were virtually unreinforced. At this time we were attempting to use uncharged membranes and found that available reinforcing materials all carried significant negative charges. It proved impossible to make gaskets which did not injure the membranes and we devised an apparatus consisting of a series of spherical bowls, which rested in spherical gauze separators. A stack of such bowls and separators could be of any desired number. Liquids were introduced and removed from between the membranes by means of flattened polythene tubes. The liquids were syphoned out into the heat exchangers, and pumped back from the heat exchangers into the apparatus.

After we had developed the technique of making amphoteric membranes the charge on the reinforcing fibres could be balanced, and it was possible to use reinforced membranes strong enough to be held satisfactorily using gaskets. We have employed an apparatus constructed of polymethyl methacrylate, using neoprene washers as gaskets, see Fig. 1. A large apparatus built on these principles would be expensive to construct and clumsy to assemble.

For large-scale work the design shown in Fig. 2 is preferable, since only plain rectangular membranes and spacers are required, with strips of rubber as gaskets. It should be very easy to assemble, and disassemble, and plumbing arrangements should be straightforward.

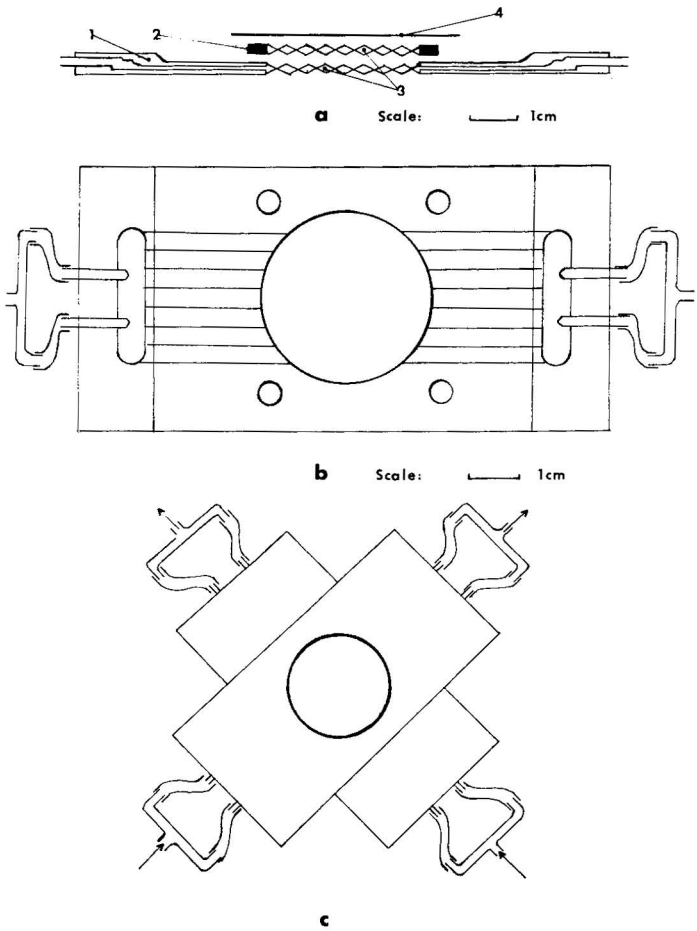


Fig. 1. Apparatus used in small experiments. (a) Exploded section through single compartment with polymethyl methacrylate scale containing flat flow tubes (1), neoprene washer (2), plastic gauze separators (3), isoelectric membrane (4). (b) Plan view of polymethyl methacrylate separator. (c) Method of assembly, but with electrode compartment omitted.

3. THE ELECTRODE COMPARTMENTS

It is essential to avoid unwanted reactions at the electrodes. Most buffers undergo either oxidation at the anode and/or reduction at the cathode. The simplest way of avoiding these is to separate the electrodes from the rest of the apparatus by efficient (dense and with high concentration of charges) ion-exchange membranes. Such membranes have a low electrical resistance and show low electroendosmosis. The anode, which may be immersed in dilute sulphuric acid, is separated from the rest of the apparatus by a cation-exchange membrane containing a high concentration of sulphonic acid residues. The only cation present in the anode compartment is then the hydrogen ion and it carries the whole of the current through the ion-exchange membrane.

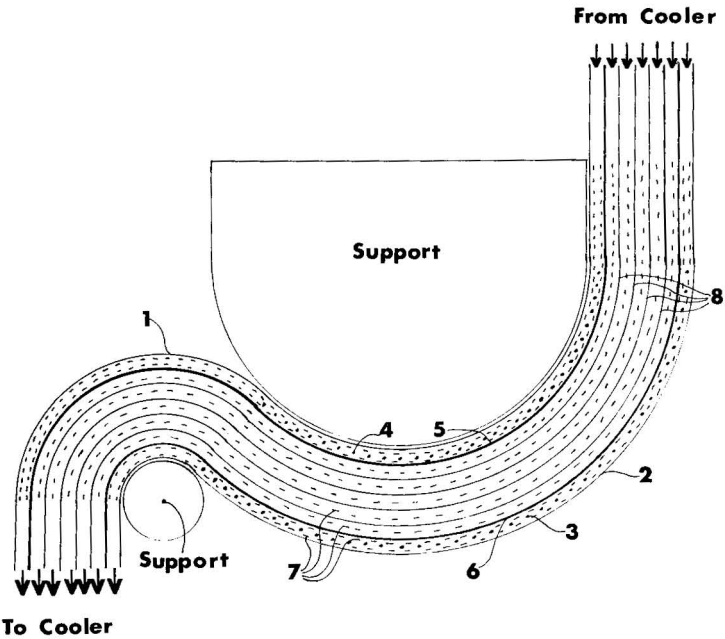


Fig. 2. Section through apparatus suggested for large-scale separations. 1 and 2 = impermeable plastic sheets stretched over formers to hold the sandwich of membranes watertight; 3 = cathode; 4 = anode; 5 = cation-exchange membrane; 6 = anion-exchange membrane; 7 = gauze separators; 8 = isoelectric membranes. The rubber strip gaskets which seal the edges of the membranes and plastic sheets are not visible in this section, but would be seen in an end elevation.

On the side of the membrane away from the anode, the only ions drawn to it are anions which are excluded by the high concentration of negative charges fixed in the membrane.

Similarly, the cathode which may be immersed in sodium hydroxide, is separated from the rest of the apparatus by an anion-exchange membrane containing a high concentration of quaternary amino groups. The only anion, except for any carbonate impurity, is the hydroxyl ion which will carry virtually the whole of the current through the membrane; cations from the non-electrode side of the membrane being excluded by the high density of fixed positive charges. With sulphuric acid as the anolyte a lead electrode which becomes covered with lead peroxide seems entirely satisfactory, and the cathode immersed in sodium hydroxide solution can be made of many metals, mild steel being perfectly satisfactory.

4. PREPARATION OF AMPHOTERIC MEMBRANES

Hardened filter papers (Whatman grade 541) were soaked in hot aqueous agarose solution (4% w/w) and the surplus agarose was removed by pressing the paper between two warm sheets of plate glass. After the impregnated papers had cooled they were soaked for one hour in 2.5 *M* aqueous sodium hydroxide, then after blotting lightly they were suspended overnight in xylene containing 1-chloro-2,3-epoxypropane (1% v/v). The membranes were then soaked for one hour in 2.5 *M* sodium hydroxide

and immersed for exactly 2 h in an alkaline solution freshly prepared by mixing equal volumes of 5.0 *M* sodium hydroxide and 2 *M* chloracetic acid. The membranes were then rinsed in 2.5 *M* sodium hydroxide solution containing diethanolamine (0.06–0.12 *M*). The membranes were then blotted lightly and suspended overnight in xylene containing 1-chloro-2,3-epoxypropane (8%, v/v). This procedure gives membranes with isoelectric points between 4.8 and 5.5, depending on the concentration of diethanolamine used.

The ion-exchange membranes were obtained from Permutit Bobey (Brentwood, Great Britain).

5. THEORY OF AMPHOTERIC MEMBRANES

For satisfactory separation the rate of electroendosmotic flow through the membrane must be low compared with the electrical migration velocity of the wanted substance. It is impracticable to attempt to use uncharged membranes, since absorption of traces of charged compounds always ensues, and causes a large endosmotic flow. To reduce the effect of absorbed substances it is necessary for the membrane to have a relatively high concentration of appropriate charges which will make it isoelectric at a given pH, and which possesses buffering power at this pH. This ensures that absorbed ions will only change the isoelectric point by a small amount.

If such an isoelectric membrane separates two solutions in an electrophoresis apparatus, one of higher pH than the isoelectric point on its cathodic side, and one of lower pH than its isoelectric point on its anodic side, then it automatically reduces the endosmotic flow to a negligible amount. Suppose that there is a flow through the membrane towards the anode, this will cause the membrane to become more alkaline on its cathode side and hence more negatively charged. The solution in the membrane will then carry an excess of positive charges and will be urged by the electric field to move in a cathodic direction. This will cause the flow to reduce until the charges on the anode side of the membrane become more positive. A similar argument applies to flow in the opposite direction. The flow will continue until the electroendosmotic pressure balances the applied difference of pressure. If the pH difference across the membrane is reversed the system is unstable and the electroendosmotic flow will continue in the direction in which it started when the current was first applied.

The membrane may be regarded as a large multivalent molecule, and has the property of all such molecules in spreading the pK values of a given type of dissociating group so that it will buffer over in much wider range than the same group would in free solution. It is possible to adjust the isoelectric point of a membrane by the addition of strong non-buffering ions such as sulphonic acid or quaternary ammonium groups without losing buffering capacity at the isoelectric point. This process is, of course, analogous to that described by Svensson for carrier ampholytes, and as with carrier ampholytes, the membrane will be conducting.

The membranes must obviously be permeable to the substances to be separated and for use with proteins, this limits the choice of material to porous gels such as cross-linked polyacrylamide or agarose, or other gels where the long chains are in contact, leaving free space between them. Such gels are inconveniently weak mechanically, but may be reinforced by stronger material with very much larger pores. Contrary to the difficulties this brings with uncharged membranes, the charge on the

surface of the supports is unimportant if they are filled with buffering gels. The pore size must be large enough not to make a substantial difference to the rate of migration of the substances being separated. If large molecules with high mobility in free solution are very much slowed down by the membrane, then a high-concentration layer will build up against the surface and the charge properties of this layer will come to dominate the properties of the membrane. The farther a substance is from its isoelectric point the larger is its charge and its mobility. The change of charge with pH could be derived from the titration curve of the protein, but cannot in general be predicted.

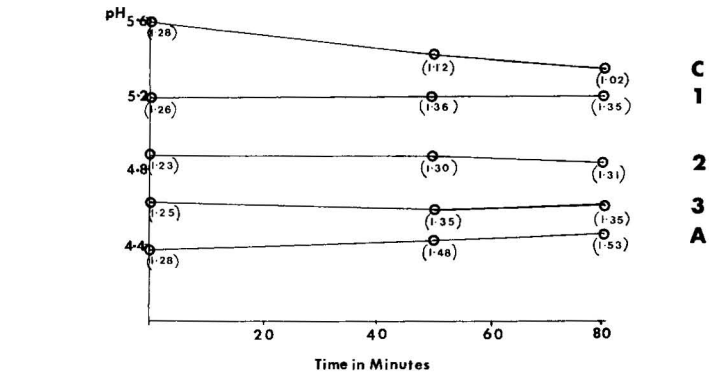
The rate of movement of molecules through the membrane is the algebraic sum of their own mobilities and of any residual electroendosmotic flow, so that the exact pH at which a substance stays within a compartment will be slightly influenced by any small electroendosmotic flow. The constancy of these factors rather than simple diffusion, which seems to be very slow, will limit the resolution of the method. Inevitably, if the difference in isoelectric point between two substances is very small, their separation will be very slow.

As a corollary, the temperature must be maintained constant, since a change will not necessarily have the same effect on the isoelectric points of the two proteins and of the membranes.

If an ideal uncharged membrane were available, it would be possible to place this between either the anion or the cation-exchange membrane and the rest of the apparatus, and then circulate the liquids between the compartments next to the ion-exchange membranes protecting the anode and cathode so rapidly that the composition would be identical at each end. In this situation where the pH on the anodic side of a membrane is higher than the pH on its cathodic side we have not been able to produce an ideal uncharged membrane. If one were available it would have no effect on the pH or conductivity of the liquids on either side of it. Using a Visking membrane at an acid pH we hoped to minimise the effects of the carboxylic acid groups present in it, in fact, however, slow changes occurred consistent with the membrane having a negative charge. The result of the experiment is shown in Fig. 3, where the membrane is included with a set of amphoteric membranes. This shows the effect of the different transport numbers in the Visking membrane which passes more sodium than amphoteric membranes, but the pH and conductivity changes are confined to the compartments separated by the Visking membrane and the one in circulation with it.

6. MAINTAINING A STABLE SET OF pH STEPS WITH A BUFFER SOLUTION CONSISTING OF MONOVALENT IONS

If the transport numbers of the buffer ions are the same at every membrane, then the requirement for stability is that the concentrations of both positive and negative ions in the end compartments be maintained constant. The simplest way to achieve this is to flow buffer solution of the correct concentration through each compartment at such a rate that its concentration is not sensibly changed during its passage through the apparatus. This will, of course, use a large amount of buffer solution. However, at the beginning of a purification this will serve to wash out of the apparatus charged substances showing no isoelectric point, or whose isoelectric point is outside the pH range employed in the experiment. If the wash buffer effluents from both end



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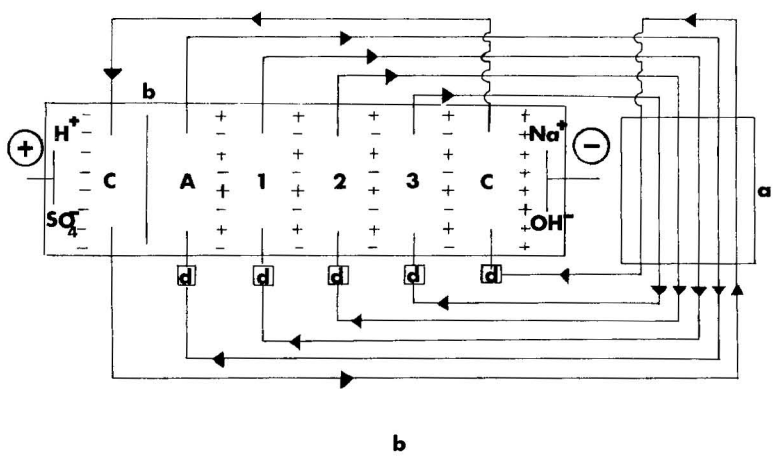

b

Fig. 3. The circulation of ions through a compartment separated by a Visking membrane. (a) Variation of pH with time in the individual compartments, the figures in brackets are the conductivities of the solutions in Ω^{-3} cm^{-1}. The starting concentration of sodium ions was 0.02 *M* in each compartment and the current was 1 A. (b) Diagram of the apparatus used. The amphoteric membranes are represented as + − + − +, the anion-exchange membrane as + + + +, the cation-exchange membrane as − − − −. a, Coolers and pumps; b, Visking membrane; d, external reservoirs.

compartments are mixed, then the conductivity of the mixture will be higher when added salts are being removed from the apparatus than when they have all disappeared, when the conductivity will fall to the value obtaining before the substance to be separated was introduced. If the buffer solutions in the end compartments are to be kept to a fixed volume and to be recirculated then the condition for stability is simply that cations must be transferred from the most cathodic compartment at the correct rate to the most anodic compartment; at the same time anions must be transferred at the correct rate from the most anodic compartment to the most cathodic compartment. The correct rate for each ion depends on the total current passing through the apparatus and on the proportion of the current carried by the particular ion, *i.e.*, on

its transport number. Some of the ions are converted to the unionised form of each buffer molecule by the H^+ and OH^- ions from the electrodes. These are not, of course, moved by the electric field and can pass through a membrane only by diffusion or electroendosmosis, which is negligible compared to the electrical transport of the ions. They are pumped along with the ions and maintain the appropriate balance.

If the hydroxyl or hydrogen ion concentration is appreciable, then the condition that the transport numbers of the buffer ions remain constant is violated and a stable system cannot be maintained without additional electrodes.

A satisfactory method of maintaining stability is to return the ions by circulation of buffer solution between the end compartments. As has been explained previously, a rapid circulation of liquids is maintained between each compartment, a heat exchanger, and a reservoir. If liquid is circulated from one end reservoir to the other end reservoir and back at the same rate, it is possible to adjust the concentrations of buffer solutions in each reservoir, such that the rate of transfer of ions by the electric field can be exactly balanced by the rate of transfer by circulation. We propose to call this return of ions by circulation "counterflow". The relation between rate of counterflow f (ml/min), the composition of the end compartments and the current I (A) for a buffer consisting of a weak acid HA and a weak base B is:

$$f(A_a^- + HA_a + BH_a^+ + B_a - A_c^- - HA_c - BH_c^+ - B_c) = \frac{I}{F}\left(T_{A^-} - T_{BH^+}\right)$$

where A_a^- is the concentration of A^- in the anodic end compartment, BH_c^+ is the concentration of BH^+ in the most cathodic compartment, and so on. T_{A^-} and T_{BH^+} are the transport numbers of A^- and BH^+, respectively. The pH of the most anodic compartment is then:

$$pH = pK_{HA} + \log A_a^- - \log HA_a$$

$$= pK_B + \log BH_a^+ - \log B_a$$

and of the most cathodic compartment:

$$pH = pK_{HA} + \log A_c^- - \log HA_c$$

$$= pK_B + \log BH_c^+ - \log B_c$$

The simplest way of providing a counterflow with two equal rates of flow is to pump in one direction and allow the return to be over a weir, which is practicable since the electroendosmotic flows are very small.

The effect of this type of circulation is illustrated in Fig. 4. It will be noted that the pH is very much more stable in the inner compartments than in the most cathodic. The reservoir for the most anodic compartment was not easily accessible and no measurements were made of its composition during the experiment. Most of the changes will have been due to temperature rise.

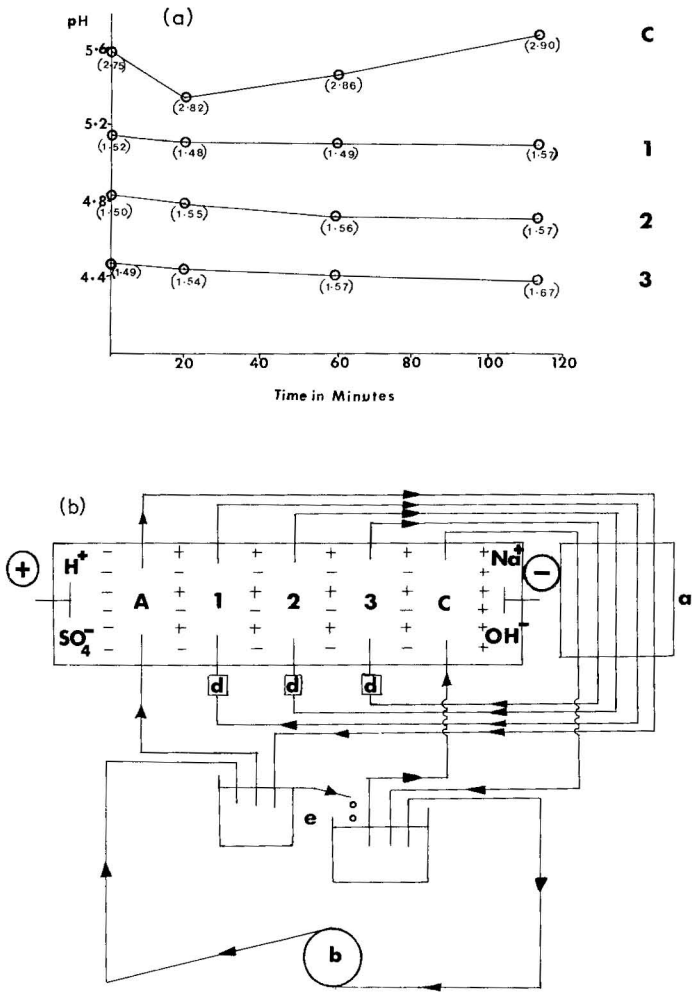

Fig. 4. The use of "counterflow" to recirculate ions. (a) Variation of pH with time in the individual compartments; the figures in brackets are the conductivities of the solutions in Ω^{-3} cm^{-1}. The concentration of sodium ion at the start was 0.02 *M* in compartments A, 1, 2, and 3 and 0.03 *M* in compartment C, the current was 1.0 A and the rate of counterflow was 18.1 ml/min. (b) Diagram of the apparatus. The amphoteric membranes are represented as $+-+-$, the anion-exchange membrane as $++++$, the cation-exchange membrane as $----$. a, pumps and coolers; b, metering pump; d, external reservoirs; e, weir for the counterflow circulation.

7. CONCLUSION

As yet sufficient work has not been done to indicate how good a separation can be achieved in this apparatus, but we feel confident that for preparative purposes it will be adequate. We regard as particularly important the probability that there is virtually no limit to the scale on which such apparatus may be constructed and operated.

8. ACKNOWLEDGEMENTS

We acknowledge with thanks the support of the Medical Research Council. U.K. Patent applications 25043/77 and 25044/77 have been made, rights in which are assigned to the National Research Development Corporation.

9. SUMMARY

A method is described for isoelectric focussing using a buffer solution containing univalent ions only. Multi-compartment apparatus is described, the compartments being separated by isoelectric amphoteric membranes. The liquid in each compartment is circulated through external heat exchangers. Current is fed into the apparatus through an anode immersed in sulphuric acid and protected by a cation-exchange membrane and by a cathode immersed in sodium hydroxide solution and protected by an anion-exchange membrane. Various means for maintaining a stable system are described, including a metered circulation of electrolytes between the end compartments, to balance the electrical flow of ions.

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PARTITION BETWEEN POLYMER PHASES

PER-ÅKE ALBERTSSON
Department of Biochemistry, Chemical Centre, University of Lund, Lund (Sweden)

CONTENTS

1. INTRODUCTION

By using aqueous polymer phase systems, it is now possible to apply partition to the analysis and separation of biological macromolecules and cell particles[1,2]. The phase systems are obtained by mixing aqueous solutions of two or more water-soluble polymers, so that all of the phases are rich in water. To these are added salts and sugars to provide a suitable environment for fragile biological particles. If two polymers are used, a two-phase system is obtained, if three polymers are used a three-phase system is obtained, etc.

This paper summarizes some of the factors that determine partition and some recent applications are considered. In most of the work carried out so far, the dextran–polyethylene glycol–water two-phase system has been used; dextran is in the lower phase and polyethylene glycol in the upper phase[1]. A large number of different biological materials have been partitioned in this system, including proteins, nucleic acids, viruses, mitochondria, chloroplasts, cell membranes and whole cells. Protein and nucleic acids are soluble in the polymer phases and their partition coefficient (concentration in the upper phase/concentration in the lower phase) depends on the molecular weight, charge, conformation and type of polymer used in the phase system. Particles such as cells and cell organelles distribute between the two phases and the interface. The latter has a large capacity for adsorption of particles and can be used for selective adsorption of particles which distribute according to their surface properties.

Much data on the behaviour of proteins and cell particles in two-phase polymer systems is now available and the main factors that determine partition can now be formulated. Partition of proteins, for example, depends upon their molecular weight, charge and probably also the kind of amino acid side-chains that are located on the surface of the proteins.

2. ELECTRICAL POTENTIAL BETWEEN PHASES

The effect of salts on the partition of charged macromolecules is dramatic. Minor changes in the ionic composition can transfer DNA almost completely from one phase to the other. It is mainly the kind of ions present and the ratio between different ions that determine the partition of biopolymers. The ionic strength is not so important. This can be explained by an electrical potential between the two phases. The potential is created by an unequal affinity of ions for the phases. Careful studies on the partition of inorganic salts in a dextran–polyethylene glycol–water system have shown that different salts have small but significant differences in their partition coefficient[3].

Such partition differences between salts mean that the different ions have different affinities for the two phases. Hence an electrical potential difference between the phases is created[1]. For a salt, the ions of which have charges Z^+ and Z^-, the interfacial potential, ψ, is given by

$$\psi = \frac{RT}{(Z^+ + Z^-)F} \cdot \ln (K_-/K_+) \tag{1}$$

where R is the gas constant, F is the Faraday constant, T is the absolute temperature and K_- and K_+ are the partition coefficients of the ions which they would have if ψ could be set to zero, *i.e.*, K_- and K_+ are expressions of the affinities of the ions for the two phases due to forces except electrical. The interfacial potential will be larger the larger is the K_-/K_+ ratio, *i.e.*, a salt with two ions that have very different affinities for the two phases will generate a larger potential difference than a salt with ions that have similar affinities for the two phases.

Further, it can be shown[1] that in the presence of excess of salt a protein will partition according to

$$\ln K_p = \ln K_p^\circ + \frac{ZF}{RT} \cdot \psi \tag{2}$$

where K_p is the partition coefficient of the protein, K_p^0 is the value of this coefficient when the interfacial potential, ψ, (generated by the excess of salt) is zero or when the protein net charge, Z, is zero. Hence, the difference in partition of the ions of the salt generates an electrical potential difference according to eqn. 1, which in turn affects the partition coefficient of the protein according to eqn. 2. Even if ψ is small, it will strongly influence K_p, because Z is large for most proteins and K changes exponentially with Z. The same holds for other charged macromolecules and more so for cell particles, which carry a vast number of charges per particle.

Experimental data on proteins fit eqn. 2 fairly well. Proteins were partitioned in dextran–polyethylene glycol systems at different pH and with different salts[4–6]. A plot of log K against the net charge of the protein gives straight lines (Fig. 1), as expected from eqn. 2. The slopes of the lines are different for the different salts used as these generate different values of ψ. The different lines intersect the ordinate at approximately the same log K_0 value.

If K_0 in eqn. 2 is assumed to be independent of pH, then the ψ value can be

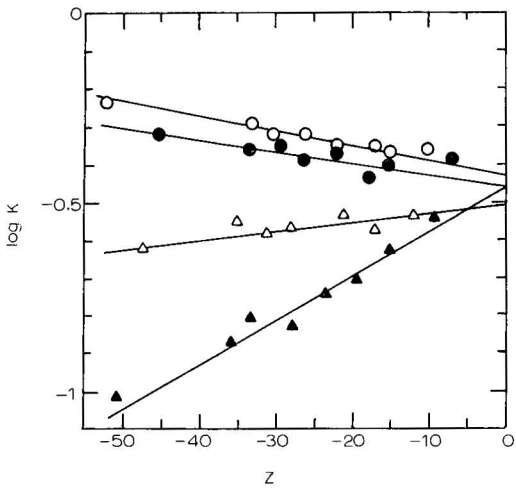

Fig. 1. Partition of a protein (bovine serum albumin) as a function of its net charge in four different phase systems having different interfacial electrical potentials[4]. Compare eqn. 2. Phase systems: 5% dextran, 4% polyethylene glycol and ○, 0.05 *M* K_2SO_4; ●, 0.1 *M* LiCl; △, 0.1 *M* KOAc; ▲, 0.1 *M* KCl.

calculated from the slope of lines in Fig. 1. The interfacial potentials thus obtained are in the range 0–5 mV. Other measurements using electrodes give similar values[7]. If the interfacial potential of a phase system is known, we can use partition in this system for the determination of the net charge per molecule of an unknown substance, without knowing its molecular weight. In this way, the net charges of different isoenzymes of enolase have been determined in an extract of yeast[8].

It is striking that the interfacial potential with a given salt varies very little with ionic strength. Further, partition of protein in a given salt does not depend significantly upon ionic strength in the range 5–100 m*M*. That is, for a given ψ, the effective net charge of the protein, Z, is independent of ionic strength. This behaviour is in contrast to other physical phenomena where charge is involved, such as electrophoresis and ion-exchange chromatography; electrophoretic mobility and adsorption on an ion-exchange column depend strongly on ionic strength.

Phase systems with zero interfacial potential can be constructed by choosing a suitable salt or salt mixture. Partition of proteins in such a system should be independent of the net charge of the protein, *i.e.*, independent of pH. Some proteins also show a constant partition coefficient over a wide pH range in zero-potential phase systems[4–6]. Other proteins show changes in certain pH ranges. In some instances this occurs when the protein undergoes a conformational change, such as in serum albumin at low pH or when protein molecules form dimers, as with lysozyme. For such proteins, K_0 is not independent of pH and partition in zero-potential systems could therefore be used to detect conformational changes when, for example, previously hidden groups are exposed on the protein surface, or to study association–dissociation phenomena among proteins.

3. HYDROPHOBIC INTERACTIONS

The electrical effects can be nullified by choosing a suitable salt composition of the phase system. For such systems, other factors that determine the partition come to the fore, such as the hydrophobic–hydrophilic balance of the particle surface and conformation.

We can split the partition coefficient into several factors such that the logarithm of the partition coefficient is the sum of several more or less independent terms:

$$\ln K = \ln K_{el} + \ln K_{hfob} + \ln K_{hfil} + \ln K_{conf} + \ldots \quad (3)$$

where K_{el}, K_{hfob}, K_{hfil}, K_{conf} represent partition coefficient factors depending on electrical, hydrophobic, hydrophilic and conformational effects, respectively. $\ln K_{el}$ is zero when the interfacial electrical potential is zero. It should be possible to increase the hydrophobic effect on partition by binding hydrophobic groups on the polymers or by including a detergent that interacts with the hydrophobic parts on a protein molecule. Several non-ionic detergents contain a polyethylene glycol chain as the hydrophile. Micelles of these detergents expose polyethylene glycol chains on their surface and, therefore, prefer the polyethylene glycol-rich phase of, for example, the dextran–polyethylene glycol system. Likewise, proteins that bind such detergents should acquire an increased affinity for the polyethylene glycol phase (see Fig. 2). Phase systems containing detergents have been used for purifying a membrane-bound enzyme, phospholipase A, from *Escherichia coli* according to this principle[9]. By using detergents with different hydrophobic parts, it might be possible to achieve selective extractions of hydrophobic proteins or membrane fragments having different hydrophobic regions.

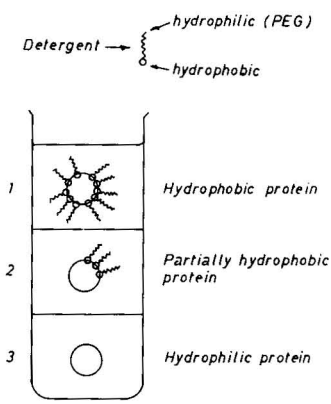

Fig. 2. Hydrophobic affinity partition. A detergent that binds to a hydrophobic protein will selectively extract this protein to phase 1, which has an affinity for the hydrophile of the detergent[9].

Digitonin is another type of detergent, the hydrophilic part of which consists of sugar units, and the micelles of digitonin prefer the lower dextran phase of the dextran–polyethylene glycol system. Therefore, proteins or membrane fragments that bind the hydrophobic part of digitonin would expose a surface of polysaccharide character and be enriched in the lower dextran phase. One could therefore imagine

mixtures of membranes with digitonin and polyethylene glycol detergents where differential binding to different membrane fragments would cause them to separate between two phases.

Recently, large hydrophobic groups, such as fatty acid chains, have been covalently bound to polyethylene glycol or dextran, and their effect on partition has been studied[10–12]. Proteins such as serum albumin, which are known to interact with fatty acids are strongly affected in their partition behaviour. Thus, palmitoyl polyethylene glycol selectively extracts serum albumin from plasma into the upper phase, while almost all of the remaining proteins remain in the lower phase[10].

Partition of cell organelles and cells is strongly influenced by hydrophobic groups attached to the polymers. Palmitoyl polyethylene glycol at concentrations as low as 0.0001–0.01 % may thus transfer chloroplasts, membranes or red blood cells from the lower phase or the interface into the upper phase. Some selectivity in the effect of the hydrophobic groups has been demonstrated; *e.g.*, deoxycholate esterified to polyethylene glycol was more effective than palmitoyl polyethylene glycol in separating two different chloroplast particles having different surface membranes[13].

Saturated and unsaturated fatty acids attached to polyethylene glycol were used for studying the surface hydrophobic properties of red cells[14]. Large differences in partition behaviour for cells from different species and also in the effect of different fatty acids were found. These could be correlated to the phosphatidylcholine and sphingomyelin composition of the different red cell membranes. As these phospholipids are located mainly on the outer layer of the phospholipid bilayer, the results indicate that the fatty acids attached to the polyethylene glycol, upon binding to the cells, interact with the outer layer of the red cell membrane.

Similar studies on the partition of liposomes, composed of different phospholipids, demonstrate that the polar head group of the phospholipid has a strong influence on the partition[15].

4. BIOSPECIFIC INTERACTIONS —AFFINITY PARTITION

By binding a biospecific ligand covalently to one of the phase polymers, it should be possible to achieve biospecific partition in a similar fashion to affinity chromatography. Recent experiments have demonstrated that such "affinity partition" is feasible. The ligands used include enzyme inhibitors for selective partition of enzymes[16], fatty acids for selective extraction of serum albumin[10], dinitrophenyl for selective partition of S-23 myeloma protein[17] and steroid for selective partition of a steroid enzyme[18]. Of particular interest is the fact that affinity partition can be applied to membranes. Thus, ligands which bind to membrane receptors were coupled to polyethylene glycol and used for the selective partition of membrane vesicles from the electrical organ of a fish[19].

5. SOME RECENT APPLICATIONS

5.1. Chloroplasts

A very complex and heterogeneous mixture of particles is obtained when a cell is disintegrated, for example by mechanical homogenization. By centrifugation, such a mixture can be separated into fractions that contain particles with the same size or

density. Such centrifugal fractions are still very heterogeneous, however, and further separation is often desirable. This can be achieved by partition, which separates according to surface properties and therefore complements centrifugation.

A conventional chloroplast preparation, obtained by differential centrifugation, can thus be separated into at least three different populations by counter-current distribution[20]. The chloroplast particles of the three populations differ to a great extent in surface properties, protein : chlorophyll ratios, ultrastructure and metabolism, but very little in size. One population consists of intact chloroplasts surrounded by the chloroplast envelope; the second population consists of chloroplasts that have lost their envelopes; and the third population consists of particles containing intact chloroplasts surrounded by a membrane-bound cytoplasmic layer including mitochondria and peroxisomes.

Another example in which partition together with centrifugation has yielded further purification is the recent separation of "inside-out" from "outside-out" vesicles from disintegrated chloroplast membranes[21]. By treatment with a high-pressure cell, the photosynthetic chloroplast membranes are fragmented into small vesicles. Such a population of vesicles consists of a whole spectrum of vesicles differing in size, chemical composition and photosynthetic activity. By differential centrifugation it can be separated into various size classes of vesicles. These centrifugal fractions can be further separated by partition into classes of vesicles that have different surface properties (see Figs. 3 and 4).

Analyses of these fractions have shown that partition separates vesicles that are turned inside-out from "outside-out" vesicles. Thus, a combination of two separa-

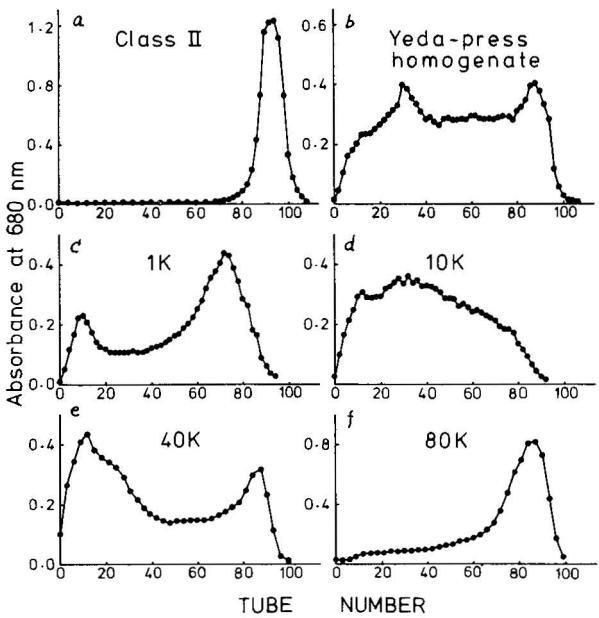

Fig. 3. Counter-current distribution diagram of (a) class II chloroplasts and (c), (d), (e) and (f), different centrifugal fractions of the Yeda press-treated material, (b). 1K = 1000 *g*, 10 min; 10K = 10,000 *g*, 30 min; 40K = 40,000 *g*, 30 min; 80 K = 80,000 *g*, 60 min.

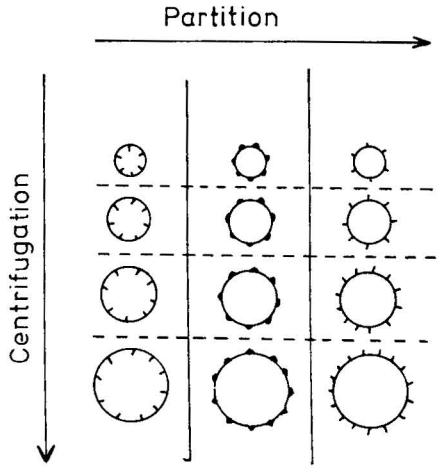

Fig. 4. Schematic diagram of membrane vesicles having different sizes and surface properties. Centrifugation will separate this mixture mainly into size classes, *i.e.*, along the broken lines, while partition separates mainly according to surface properties, *i.e.*, the full lines. A combination of these two techniques will therefore achieve a better separation than either of them alone.

tion techniques, centrifugation, which separates according to size, and partition, which separates according to surface properties, has considerably increased the separation and allowed, for the first time, the isolation of inside-out chloroplast vesicles.

5.2. Determination of hydrophobicity of water-soluble proteins

A method for measuring the hydrophobicity of water-soluble proteins has recently been described[11]. It involves partition of proteins in a dextran–polyethylene glycol system where some of the polyethylene glycol is esterified with fatty acids. Proteins that interact with hydrocarbon groups will partition more in favour of the upper polyethylene glycol-rich phase. The difference, $\Delta \log K$, between the logarithm of the partition coefficient of the protein in a system with and without fatty acid ester is taken as a measure of the hydrophobicity. In this way, interactions other than hydrophobic are eliminated. Fig. 5 shows a plot of $\Delta \log K$ against percentage of palmitoyl polyethylene glycol for six different proteins: serum albumin, β-lactoglobulin, haemoglobin, myoglobin, ovalbumin and α-chymotrypsinogen. As expected, serum albumin and β-lactoglobulin show considerable hydrophobicity. At 5% palmitoyl polyethylene glycol, $\Delta \log K$ for serum albumin is about 2 and for β-lactoglobulin it is about 1.5. Less expected is the finding that haemoglobin and myoglobin also display hydrophobicity. Myoglobin has a $\Delta \log K$ value that is about one quarter of that of a haemoglobin, *i.e.*, the same ratio as for their molecular weights. Ovalbumin and α-chymotrypsinogen show no detectable hydrophobicity. Similar studies can be carried out using other hydrophobic groups in the polyethylene glycol.

Because factors other than hydrophobic interactions are eliminated by this type of "difference partition", and because the proteins are not denatured, the $\Delta \log K$ value can be taken as a practically useful estimate of hydrophobicity. It allows for the first time a well defined comparison of hydrophobicity between different water-soluble proteins.

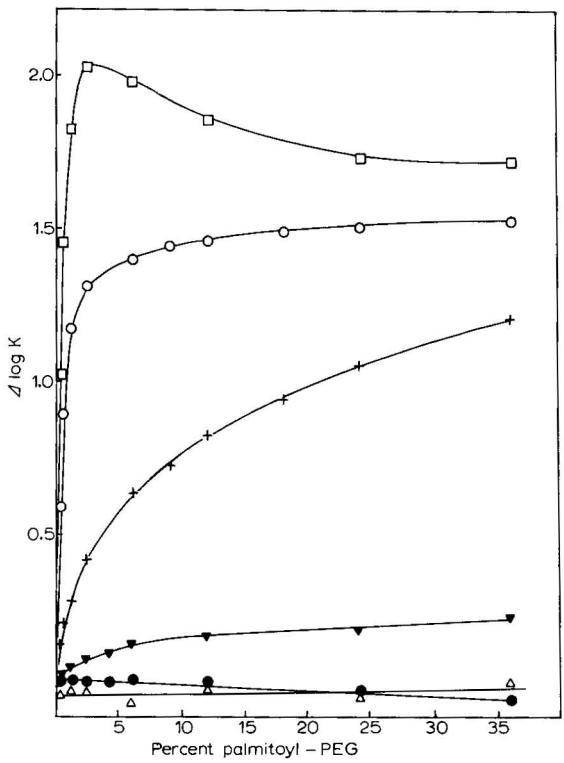

Fig. 5. Proteins partitioned in two dextran–polyethylene glycol two-phase systems, one with and the other without palmitoyl groups bound to the polyethylene glycol. The difference in log K between the two systems is plotted against the degree of substitution of palmitoyl. Δ log K can be taken as a measure of the hydrophobicity of the proteins. Proteins: □, serum albumin; ○, β-lactoglobulin; +, haemoglobin; ▼, myoglobin; ●, x-chymotrypsinogen; △, ovalbumin.

The method has recently been applied for comparing the hydrophobic properties of different histones[22].

6. INTERACTING MOLECULES

When a pair of interacting molecules are partitioned together, the overall partition should be different than that which occurs when the respective compounds are partitioned alone.

Suppose, for example, that the partition coefficient of a substance A is K_A and that of another substance B is K_B. If there is no interaction between A and B, the same partition of A will be found whether B is present or not. Likewise, the presence of A will not influence the partition of B. However, if A and B interact in some way, the presence of A will perturb the partition of B and *vice versa*. We might assume, for example, that A and B form a complex, AB, the partition coefficient of which is different from both K_A and K_B. The different equilibria in such a case are shown in Fig. 6. There are two association equilibria, one for each phase, and three partition equilibria. If the total concentrations of A and B can be determined in the two phases,

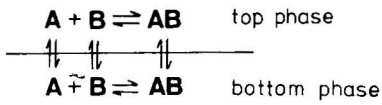

Fig. 6. Partition of two interacting molecules giving rise to a 1:1 complex in each phase.

the equilibrium constants can be calculated. In this manner, interactions between molecules can be detected and also studied quantitatively.

We use the following symbols to denote concentrations, partition coefficients and dissociation constants:

$[A^\circ]_t$ = total concentration of A in top phase;
$[B^\circ]_t$ = total concentration of B in top phase;
$[A]_t$ = concentration of free A in top phase;
$[B]_t$ = concentration of free B in top phase;
$[AB]_t$ = concentration of complex AB in top phase.
By replacing the subscript t with b, corresponding symbols for the bottom phase are obtained.
K_A, K_B, K_{AB} = partition coefficients for A, B and AB, respectively.
K_t, K_b = dissociation constants in the top and bottom phase, respectively.

The following equations can be written:

$$K_A = \frac{[A]_t}{[A]_b} \tag{4}$$

$$K_b = \frac{[B]_t}{[B]_b} \tag{5}$$

$$K_{AB} = \frac{[AB]_t}{[AB]_b} \tag{6}$$

$$[A]_t + [AB]_t = [A^\circ]_t \tag{7}$$

$$[B]_t + [AB]_t = [B^\circ]_t \tag{8}$$

$$[A]_b + [AB]_b = [A^\circ]_b \tag{9}$$

$$[B]_b + [AB]_b = [B^\circ]_b \tag{10}$$

$$K_t = \frac{[A]_t\,[B]_t}{[AB]_t} \tag{11}$$

$$K_b = \frac{[A]_b\,[B]_b}{[AB]_b} \tag{12}$$

If we take out a sample from the top phase and dilute it such that the complex AB dissociates and suppose that we then can assay A and B separately, for example by an enzymatic, immunological or radioactive assay, then we can determine the total

concentration of A and B in the upper phase. In the same way, the total concentration of A and B in the bottom phase can be determined. Thus, $[A^\circ]_t$, $[B^\circ]_t$, $[A^\circ]_b$ and $[B^\circ]_b$ will be known. K_A and K_B can be determined by measuring the partition coefficients of the proteins separately. The remaining nine unknowns can be solved by means of the above equations. We obtain the following relationships for the dissociation constants and the partition coefficient of the complex:

$$K_t = \frac{K_A K_B \left\{[A^\circ]_b - [B^\circ]_b - \frac{1}{K_b}([A^\circ]_t - [B^\circ]_t)\right\}\left\{[A^\circ]_b - [B^\circ]_b - \frac{1}{K_A}([A^\circ]_t - [B^\circ]_t)\right\}}{(K_B - K_A)\left(\frac{1}{K_A}[A^\circ]_t - \frac{1}{K_B}[B^\circ]_t - [A^\circ]_b + [B^\circ]_b\right)} \tag{13}$$

$$K_b = \frac{\left\{[A^\circ]_t - [B^\circ]_t - K_B([A^\circ]_b - [B^\circ]_b)\right\}\left\{[A^\circ]_t - [B^\circ]_t - K_A([A^\circ]_b - [B^\circ]_b)\right\}}{(K_A - K_B)\left(K_A[A^\circ]_b - K_B[B^\circ]_b - [A^\circ]_t + [B^\circ]_t\right)} \tag{14}$$

$$K_{AB} = K_A K_B \cdot \frac{\frac{[B^\circ]_t}{K_B} - \frac{[A^\circ]_t}{K_A} + [A^\circ]_b - [B^\circ]_b}{K_A[A^\circ]_b - K_B[B^\circ]_b - [A^\circ]_t + [B^\circ]_t} \tag{15}$$

Hence the dissociation constants and the partition coefficient of the complex can in principle be determined by one partition only.

It is assumed that only a 1:1 complex between A and B is formed. Also, the partition coefficients K_A and K_B must be different. If they are only slightly different the method is not very accurate and if they are identical the calculation cannot be used.

The partition coefficient of the complex, K_{AB}, can also be determined if an excess of A over B is added to the system. If the excess is so large that all B is in the complex, its partition coefficient can be determined.

The following expression for the dissociation constant in the bottom phase can then be written:

$$K_b = \frac{([A^\circ]_b - \varphi)([B^\circ]_b - \varphi)}{\varphi} \tag{16}$$

where

$$\varphi = \frac{[A^\circ]_t - K_A[A^\circ]_b}{K_{AB} - K_A}$$

and the dissociation constant in the top phase will be

$$K_t = \frac{K_A K_B}{K_{AB}} \cdot K_b \tag{17}$$

In this instance K_A and K_B may be similar, provided that they are different from K_{AB}.

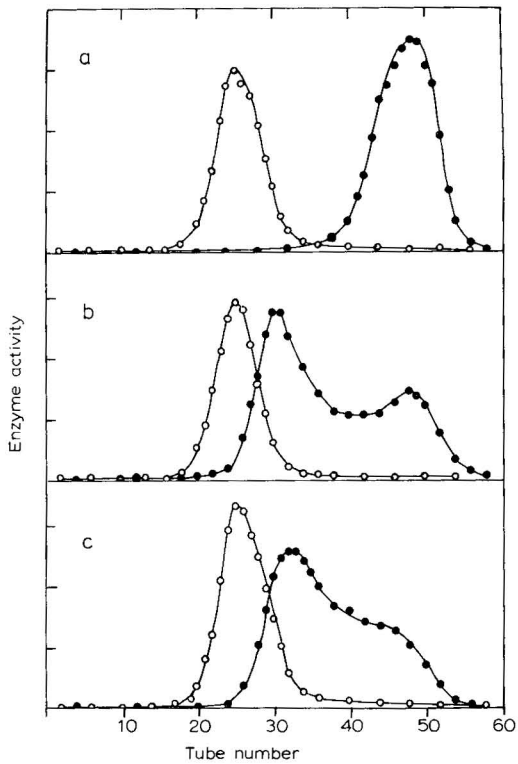

Fig. 7. Detection of interaction between two enzymes, aspartate aminotransferase (○) and malate dehydrogenase (●), by counter-current distribution. (a) The two enzymes were run separately; (b) and (c) the two enzymes were run together with aminotransferase: malate dehydrogenase activity ratios of 12 and 24, respectively[26].

The following types of interactions have been studied: protein–small ligand[23], DNA–small ligand, ribosomes–small ligand[24], protein–DNA, RNA–RNA[1], protein–RNA[25] and protein–protein[26,27].

Of particular interest is the use of equilibrium partition for the detection of weak interactions, which are difficult to detect by other methods. An example is shown by studies on the interaction between malate dehydrogenase and aspartate transaminase[26]. These two enzymes catalyse two consecutive metabolic steps and there has been speculation about their possible physical association. Each enzyme has one cytoplasmic and one mitochondrial isoenzymic form. Using phase partition in combination with counter-current distribution, Backman and Johansson[26] first demonstrated a physical interaction between the cytoplasmic forms of malate dehydrogenase and aspartate transaminase and then also between the mitochondrial forms of the two enzymes. However, no interactions between the heterotopic enzymes was found, *i.e.*, between cytoplasmic malate dehydrogenase and mitochondrial aspartate transaminase or between mitochondrial malate dehydrogenase and cytoplasmic aspartate transaminase. Thus, each enzyme seems to recognize its appropriate neighbour enzyme. This suggests that enzymes, in addition to catalytic and regulatory sites, also expose recognition or "social" sites which interacts with neighbouring enzymes *in vivo*.

7. SUMMARY

Separation of biopolymers and cell particles can be accomplished by partition between two or more immiscible, liquid, aqueous, polymer-containing phases. The phase systems are obtained by mixing water solutions of different polymers, such as dextran and polyethylene glycol. This review describes the various factors which determine the partition. By binding a biospecific ligand covalently to one of the phase polymers, biospecific affinity partition is obtained. Applications on the separation of chloroplasts and membrane vesicles are described.

Partition can also be used to detect and study interaction between macromolecules. Formulae for the calculation of the dissociation constant for a 1:1 protein–protein complex are presented.

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LYMPHOCYTES, RECEPTORS AND AFFINITY CHROMATOGRAPHY

LESLIE HUDSON*

Department of Experimental Immunobiology, Wellcome Research Laboratories, Beckenham, Kent (Great Britain)

CONTENTS

1. INTRODUCTION

The former apparent simplicity of the immune response has been destroyed by the recognition of a bewildering array of immunologically active cells, differing not only in specificity but also in effector function and lineage. By isolation, characterisation and recombination of the cellular components of this system it has been possible to describe to a limited extent the complex interactions constituting the immune network.

The criteria by which cells can, and have been, fractionated and characterised are numerous. Fortunately, excellent current reviews are available dealing with the isolation and characterisation of immunologically relevant cells[1] and, more particularly, with the applicability of affinity chromatography to immunology[2]. Thus I wish to limit myself here to a consideration of the general approach to cell affinity chromatography and more especially to those techniques that are likely to carry forward the study of the immune response.

2. LYMPHOCYTES, RECEPTORS AND AFFINITY CHROMATOGRAPHY

In principle, the techniques available for the immunospecific fractionation of cells rely basically upon the interaction of a more or less well-defined ligand or affinity molecule with a specific receptor on the surface of the cells to be selected. The diversity in technical design is derived from the method by which cells binding to the ligand are removed from the majority of non-reactive cells. As can be seen from Fig. 1, most affinity separations are achieved on a solid-phase immunoadsorbent composed of a support matrix carrying covalently linked affinity molecules. The fluorescence-activated cell sorter, developed by Herzenberg and his colleagues[3], is one notable

* Present address: Immunology Unit, Department of Biochemistry, Jenner Wing, St. George's Hospital Medical School, University of London, Cranmer Terrace, London SW17 0RE, Great Britain.

exception to this general approach. This is clearly the most powerful tool available by which cells binding affinity molecules may be isolated directly. There are, however, sufficient advantages associated with less sophisticated systems of affinity separation to ensure their continued use.

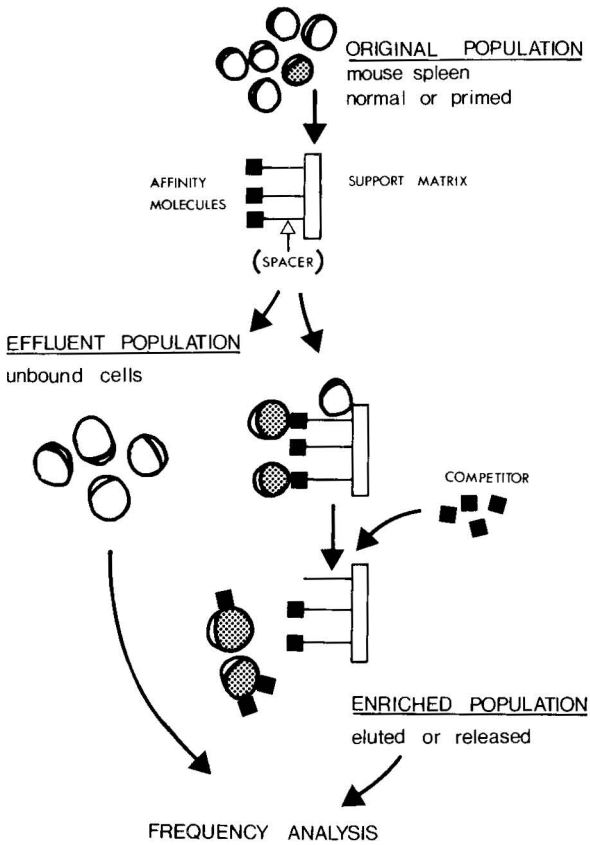

Fig. 1. Flow diagram for the selection of cells by affinity chromatography.

Immunoadsorbents with antigens or antibody[4,5], alone or in combination[6], have been used to separate lymphoid cells by adsorption via antigen-binding or other surface receptors or determinants. More recently affinity molecules with a greater chemical definition of binding specificity, for example, lectins or haemagglutinins[7] or histamine[8] have been used, although the immunological basis for ligand specificity is less clear.

The requirements of a suitable support matrix, such as ease of derivatisation, high capacity and minimal direct interaction with the cells to be selected, have led to a general adoption of cross-linked agaroses and dextrans, and to a lesser extent, acrylamide gels. In addition, the beaded presentation of these materials is advantageous because of the flow and surface area characteristics associated with beaded affinity columns[2]. Recently, Eckert *et al.*[9] has developed a beaded support by cross-

linking calf serum with glutaraldehyde. Although not as well defined as the other gels mentioned, it has the advantage of minimal non-specific adsorption of non-activated lymphoid cells.

The magnitude of interaction of cells with the affinity molecules is sufficient to retard their passage through the affinity column, but not sufficient to effect their retention. Capture of the specifically retarded cells requires interaction with the matrix, consequently, in cell affinity chromatography, specificity and capacity are somewhat antithetical.

The effect of moving the affinity molecule away from the environment of the matrix by means of a spacer or extension arm is less dramatic in cell, as opposed to molecular, affinity chromatography because of the relative cellular and molecular dimensions involved. However, spacers are available that can be degraded enzymatically[10], thermally[11] or chemically[12]. Thus the release of adsorbed cells can be effected more efficiently than by earlier techniques requiring the solubilisation of the total support matrix[13].

The release of bound cells by competition with free affinity molecules is obviously advantageous as it introduces specific desorption, as well as specific adsorption into the affinity technique. However, because of the multivalent interactions of the bound cells with the affinity column producing a very low dissociation rate, release by direct competition is not possible. A shear force is required to release the cells from the column in the presence of free competitor, this then stops re-association of the cell with the insoluble affinity molecules. Techniques are available whereby this shear force can be generated hydrodynamically[14], by centrifugation[15], by mixing[16,17] or musically[18].

Within the field of antigen-specific cell fractionation, probably the most widely studied cell population is that derived from mouse spleen containing effector or memory cells elicited by previous immunisation with the antigen to be used for affinity selection. In this case, a strict analysis of the original, enriched and depleted cell populations is possible in terms of the frequency of antibody-forming cell precursors for the antigen under test[11,19].

Much of the present research on fractionated cells is centred on the functions of the purified cells rather than on the techniques of their fractionation. It is now possible to enrich or deplete lymphocyte populations of almost any of their known cellular components. For example, using dinitrophenyl–human serum albumin (DNP–HSA) conjugated to Sephadex G200, it has been possible to fractionate spleen cell populations from mice primed to DNP_4 fowl gamma globulin (DNP_4FGG)[17]. When cells were prepared seven days after *in vivo* priming, plaque-forming cells secreting anti-DNP antibody (DNP–PFC) could be adsorbed to DNP–HSA Sephadex columns, and then recovered either by elution with DNP–lysine or by dextranase solubilisation of the matrix[13]. Although the DNP–PFC's released by DNP–lys competition were functional and showed a 30-fold enrichment over the original population, those recovered by matrix solubilisation were inactive. This disparity in findings is probably due to effector cell blockade[20]; the monovalent hapten used for elution being less efficient than the multivalent hapten released by enzymatic digestion[13]. Similar columns using DNP–lys as the affinity molecules can be used for the retention of DNP-specific memory cells obtained from the spleens of mice immunised more than six months previously with DNP_4FGG. Cells released by solubilisation of the

matrix show a 5–6-fold enrichment when assayed in a Mitchison adoptive transfer system with hapten-carrier conjugates as antigens[21]. However, a greater enrichment (10–100-fold) could be obtained when bound cells were eluted with a stepwise gradient of free DNP–lysine between 0.03 and 3.0 m*M*. Fractions of the eluted cells were assayed *in vivo* for the production of antibody-forming cells after challenge with antigens. It was found that the affinity of the antibody was inversely proportional to the concentration of DNP–lys used to elute the plasma cell precursors. The isoelectric focusing banding pattern of anti-DNP antibody showed a very limited heterogeneity within each fraction, suggesting that a relatively low number of clones had been selected. In our present work, we are attempting to use these purified memory cells in hybridisation studies. It is hoped that the relatively high frequency of antigen-specific, non-activated lymphocytes might enable us to produce hybrids responsive to antigenic stimulation.

The usefulness of this type of approach for the selection of antigen specific cells has been extended recently by Scott[16]. For example, cells binding polymerised flagellin (POL), were isolated by reacting the cell population with fluorescein(Fl)-conjugated POL. The cells reacting with Fl–POL were then isolated from the non-reactive population by filtration through a column of anti-Fl antibodies coupled to Sepharose. The column bound cells were then released by gentle mixing in the presence of Fl-bovine serum albumin.

Although the majority of procedures based on antigen-specific affinity chromatography have yielded purified B, but not T, lymphocytes, Scott[16] was able to isolate functional T effector cells by binding BALB/c cytotoxic cells to Fl-coupled EL4 lymphoma cells. The "cellular complex" was then isolated on an anti-fluorescein column and recovered as above.

The functional complexity of lymphocyte populations is by no means limited to that derived from the diversity of antigen binding receptors. It has long been known that cells capable of indirect regulation of antibody production have both positive and negative functions. It is difficult to discern the subtle attributes of those systems of affinity chromatography that have been able to isolate not only B cells but also helper T cells (positive regulators[22] and suppressor T cells (negative regulators)[12,23] apparently by direct interaction with antigen. Superficially similar systems have yielded B cells uncontaminated by T cells and therefore devoid of helper or suppressor functions[14,17].

The use of column-bound cells to adsorb unwanted antibodies from antisera[24] has recently been elegantly extended by Sela and Edelman[25]. Cells adsorbed to Con A coupled Sephadex were cross-linked with glutaraldehyde and then used as a cellular immunoadsorbent to isolate anti-carbohydrate antibody from normal sera. The potency of this technique was demonstrated by the definition of antibodies able to precipitate a glycoprotein associated with the developing, but not the growing stage of slime molds.

This technique seems an excellent tool for the analysis of cell–cell interactions. Not only should it be possible to produce antibodies to probe sites important for structure formation and ultimately organogenesis, but immobilised cells might also provide a good substrate for the isolation of receptors responsible for cell–cell recognition. Indeed, a good testing ground might be the isolation of the putative receptor for sheep erythrocytes present on human T cells.

A preliminary insight into the workings of immune lymphocyte populations has been gained by the use of various complex and relatively ill defined antigens, from hapten-carrier conjugates to sheep erythrocytes. For greater insight, more defined systems for cell fractionation will be needed, using molecules of a simplicity on the scale of the mediators of cell reactivity already known in pharmacology and endocrinology. Thus it seems likely that interest might once more cycle back to the basic systems of fractionation, because the degree of definition of cell populations is now insufficient for the increasing precision of biophysical, biochemical and immunochemical techniques. Thus, methods are required for the clonal selection and expansion of monospecific, monofunctional lymphocyte populations obtained by affinity isolation.

3. SUMMARY

The ability to isolate, characterise and recombine, in a predetermined manner, immunologically reactive cells is one of the most powerful tools with which to investigate the detailed workings of the immune network. However, the study of cell affinity chromatography centres on the cell rather than on the technique. Hence, none of the systems, with the notable exception of the fluorescence-activated cell sorter, have been subjected to extensive technological innovation. There is an obvious need for a greater characterisation of the criteria by which cells are fractionated to to improve reproducibility. These essentially biophysical techniques could then serve as a basis for defining the cell type isolated. This would remove the need to define the isolated cell only in terms of the pre-existing lymphocyte subjects, which rarely correspond directly.

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TECHNIQUES AND INSTRUMENTATION FOR PREPARATIVE IMMUNOSORBENT SEPARATIONS

J. W. EVELEIGH*
Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tenn. 37830 (U.S.A.)

CONTENTS

1. INTRODUCTION

In celebrating the 500th Anniversary of the University of Uppsala, it is appropriate to recognise that the progress I would like to present today is a direct consequence of a serendipitous discovery made within its walls by our distinguished host, Dr. Porath, a Mr. Sundberg and the chairman of this session, Dr. Kristiansen. Acting on a suggestion of Dr. Rolf Axen, they treated polymeric carbohydrates with cyanogen bromide under alkaline conditions and discovered what was to become the most widely used and successful means of preparing immobilised reagents for affinity chromatographic separations[1].

Since its discovery ten years ago, the cyanogen bromide activation of agarose has provided a ready means for the convenient synthesis of immunosorbents destined to be utilised in preparative separations of macromolecules or for associated analytical purposes. The provision of a consistently reliable spherical preparation of agarose, namely Sepharose manufactured by Pharmacia, has ensured an increasing utility of the method in comparison with alternative supports and other chemical strategies. Seven years ago our laboratory began utilising immunosorbents for the specific isolation of immunological reagents relevant to the diagnosis and understanding of human neoplastic disease[2]. The simplicity and preparative advantages offered by these biospecific methods prompted an associated research programme into the chemical and physical characteristics of the immunosorbents and in the methodology of their efficient use in preparative separation protocols. As the demand for separations and purifications increased, associated automatic instrumentation was developed to enable unattended routine processing of samples and antisera.

It is the purpose of this contribution to firstly report on some of the more significant findings in the technique of efficiently applying immunosorbents to the preparative separation of pure antigens and monospecific antibodies and subsequently to describe the evolution of the instrumentation designed for this purpose, culminating in a soft-programmable repetitive chromatograph of wide versatility, which is being

* Present address: Instruments Products Division, E.I. du Pont de Nemours, Experimental Station, Wilmington, Del. 19898, U.S.A.

introduced here publicly for the first time. In conclusion, an attempt will be made to assess two future applications of immunosorbents in improving diagnostic methods and their potential in the therapy of human disease.

The data to be presented on immunosorbent characteristics has been previously published in an internal report (ORNL-TM-4962) and is treated more fully in ref. 3.

2. CHEMICAL AND PHYSICAL CHARACTERISTICS OF SEPHAROSE BASED IMMUNOSORBENTS

Although we have investigated the potential of a wide range of support materials and activation chemistries for preparing immunosorbents, we have found that three types of support material satisfy our preparative requirements adequately. Most generally applicable are immobilised reagents prepared from cyanogen bromide activated Sepharose 4B. Where a high degree of rigidity is required for specific applications, controlled pore glass is preferable and similarly, when rapid reaction rates are desirable, solid glass spheres offer definite advantages over the porous supports. Despite many attempts to improve on the original methods of activating Sepharose[4,5], we have been generally unsuccessful and now routinely use the alkaline buffer technique of Porath *et al.*[6]. Considering the extra chemistry involved, the use of molecular extenders is hardly justified for preparative immunosorbents by the small increase in reactivity observed, particularly in comparison with other factors which will now be discussed.

In the application of antibody immunosorbents for preparative purposes, the specific reactivity of the immobilised species is an important operating parameter. Any reduction in reactivity will be reflected in a lower capacity for antigen and hence a diminished overall yield in addition to be wasteful of what may well be a valuable reagent. We have found that the specific reactivity of immobilised antibodies is inversely related to the protein density on the support. Thus, the greater the amount of antibody linked to a given volume of support, the less its specific immunological reactivity to antigen becomes. This effect is shown in Fig. 1 in which the specific reactivity, expressed as a molar ratio, of an immobilised IgG fraction of a high-titre goat anti-human albumin sera is shown as a function of increasing ligand loading.

It is apparent from this result and from similar experiments using other immunological systems that the effect is a general one and occurs on most porous supports. Using monospecific antibody preparations, the maximal reactivities achieved at low antibody densities for protein antigens are in the region of 1.20 expressed as a molar ratio. This 60% theoretical activity is reduced to less than 25% at the maximum loading on both Sepharose and porous glass; on solid matrices the effect is less pronounced, unless of course a monolayer of reagent is exceeded. A similar effect is observable with antigen-loaded immunosorbents and is probably due, in both cases, to steric hindrances accentuated by the tortuous nature of the pores within the support.

The conditions used in preparing the immunosorbents have some bearing on the loss in reactivity. The data used in Fig. 1 were obtained by limiting the degree of activation of the Sepharose by restricting the amount of cyanogen bromide used. If a constant high degree of activation is employed and the available protein restricted during the subsequent coupling reaction, there is less variation in reactivity although

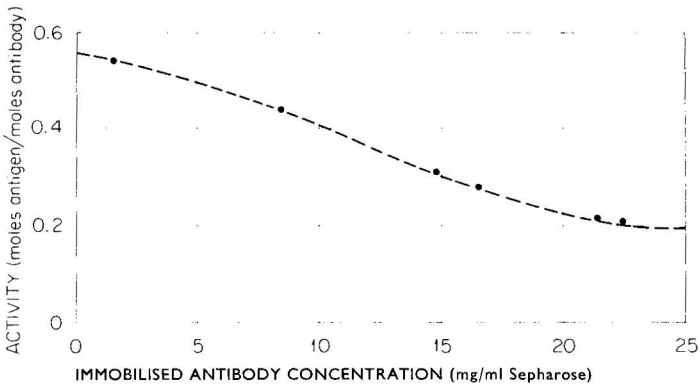

Fig. 1. Effect of increasing substitution of antibody on its immunochemical activity.

the overall values are somewhat lower in comparison with adoption of the previous strategy. These findings are of practical significance where a limited supply of reagent to be immobilised is available and maximum preparative efficiency is demanded.

Of more fundamental importance to the separational efficiency of immunosorbents is the inherent affinity constant spectrum of the antibody preparation being used. Although these equilibrium constants are many orders of magnitude greater than their equivalent counterparts in partition and ion-exchange chromatographic techniques —making the term chromatography when using immunosorbents somewhat debatable— they nevertheless dictate an equilibrium process to be considered in using immunosorbents. Their values become of significance in practical terms when very low concentrations of soluble component exist as exemplified in immunosubtractive procedures in which the removal of a trace antigen is required. In conventional preparative applications, however, the numerical value of the constants is only relevant in predicting purity of product and assessing the efficiency of eluting solutions in dissociating the immobilised complex. For these purposes it is not necessary to obtain a highly precise value, defined in chemical kinetic units, as relative values determined under similar experimental conditions are acceptable and pragmatically adequate.

Fig. 2 demonstrates the practical use of such information. This composite plot of bound antigen, expressed in convenient mass per unit volume units *versus* free antigen concentration, was constructed from values of affinity constants measured in various buffers including potential eluents. From Fig. 2, it is readily apparent that the chaotropic solutions of thiocyanate ion are considerably more effective than urea in displacing bound antigen at all free antigen concentrations. The value of this type of direct presentation, as opposed to Scatchard and other immunochemical interpretations, is that it allows ready prediction of efficiencies and thereby ultimate yields in practical terms and units. The intrinsic affinity constant of a particular antibody is of course dependent largely upon the idiosyncratic response of the immunised animal, and thus is beyond our control. However, later in this contribution the potential of antibody fractionation dependent upon affinity constants will be discussed, thus offering an alternative to this present constraint.

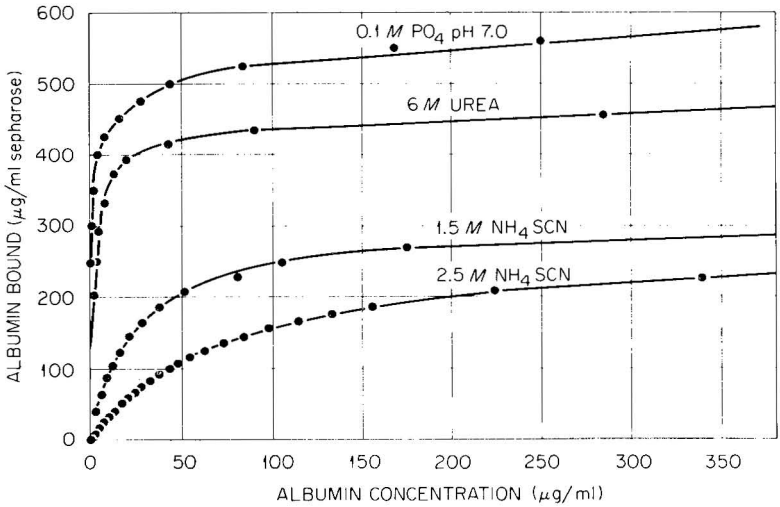

Fig. 2. "Direct plot" of albumin bound to specific immunosorbent expressed in weight per volume units as derived from affinity constants.

The largest restriction imposed by the use of immunosorbents based on porous supports is a function of the low diffusibility of macromolecules in solution. This effect is illustrated in Fig. 3 where the overall reaction kinetics of albumin with an anti-albumin Sepharose 4B immunosorbent is depicted. It is apparent that the overall reaction can be considered as biphasic; the first 80% of the reaction occurring at a faster rate than the final 20%. The finding that chaotropic dissociation, also depicted in Fig. 3, mimics the adsorption profile indicates diffusion rather than immunochemical reaction as the rate limiting parameter. This finding has significant implications in applying porous immunosorbents to preparative separations by imposing a time restriction on the process. Solid support-based immunosorbents show a much faster reaction, limited only by interstitial diffusion, but their much reduced capacity tends to negate this potential advantage.

The diffusion dependence makes its contribution felt in practical terms by lengthening the adsorption time for maximum reaction and extending wash protocols to remove contaminants. The strategy that we have evolved in overcoming these restraints is to attempt to limit reaction to the outer regions of the immunosorbent bead and thus take advantage of the more rapid reaction rate. This is most readily achieved in column operation by an increase in flow-rate and thereby limiting the exposure of sample to the surface of the immunosorbent beads and concurrently restricting contaminant diffusion into the deeper pores. The loss in effective column capacity resulting from this strategy is amply compensated by the increase in overall yield that is obtainable.

Using the automatic repetitive chromatographic systems to be described shortly, adoption of this high flow-rate procedure has resulted in a four-fold increase in yield over the earlier standard protocols. Using a 100-ml column, it has become routine practice to isolate gram quantities of monospecific antibodies of better than 95% purity on a daily basis. The quality of these products can be improved further

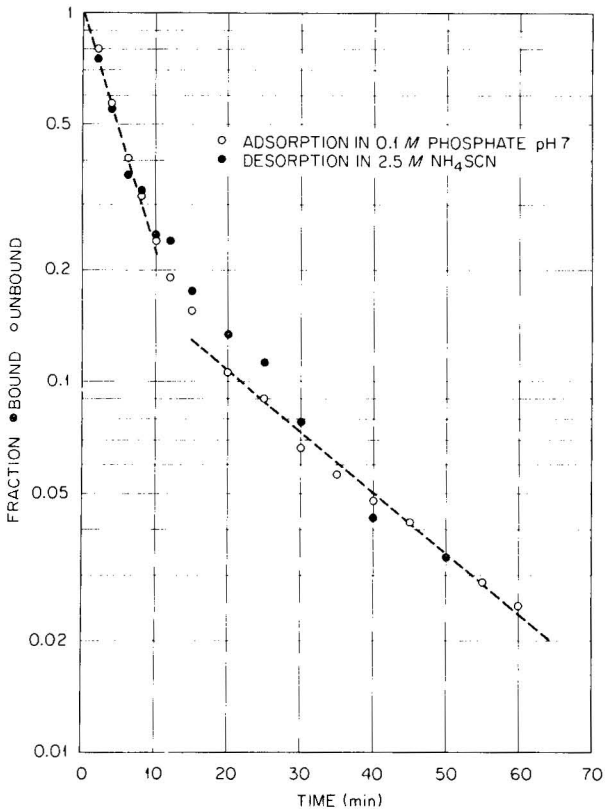

Fig. 3. Adsorption and desorption of albumin from its specific immunosorbent under stirred-batch conditions.

by incorporation of non-ionic detergents in the buffers, to reduce non-specific adsorption, and by removal of minor contaminants by reprocessing or by conventional protein chromatographic procedures.

Before describing the chromatographic systems and the associated instrumentation, there is a chemical strategy that I would like to share with you that can reduce the time and processing effort in preparing immunosorbents. This involves the reversal of an antigen immunosorbent directly into a specific antibody immunosorbent by the simple means of chemical crosslinking. The immobilised antigen is reacted with an antisera containing the specific antibodies and after washing to remove contaminating proteins is treated with a dilute solution of a divalent cross-linking agent such as glutaraldehyde or dimethyl suberimidate, the process being performed either as a batch operation or directly in a column. After a further wash with chaotrope and buffer, the unreacted groups of the cross-linking agent are blocked with an amine or hydroxylamine in the case of glutaraldehyde. The original antigen immunosorbent can now be used as specific antibody immunosorbent. Table 1 shows typical results of this reversal process as applied to albumin immobilised on Sepharose 4B; details of the procedure will be published elsewhere[7]. As one of the immunodeterminant

TABLE 1

EFFECT OF CROSS-LINKING AGENT CONCENTRATION AND REACTION TEMPERATURE ON IMMUNOSORBENT REVERSAL

1-ml samples of HSA-Sepharose were reacted for 1 h with 1.0 mg of purified goat anti-HSA antibodies and cross-linked with glutaraldehyde at pH 8.8 for 1 h.

Temperature (°C)	*Cross-linking agent conc. (M)*	*IgG cross-linked (μg)*	*Cross-linking efficiency (%)*	*Reactivity (moles/mole)*
4	0.001	380	75	0.39
4	0.005	415	80	0.51
4	0.01	445	87	0.46
4	0.02	450	85	0.46
4	0.05	415	79	0.45
25	0.001	375	83	0.52
25	0.005	380	88	0.46
25	0.01	440	88	0.46
25	0.02	455	89	0.47
25	0.05	385	85	0.47

sites of the antibody is involved in the cross-linking to antigen, the resultant reactivity is halved as can be seen in Table 2, which compares the specific reactivities obtained using this procedure with those resulting from the more conventional direct elution and immobilisation process. It is to be noted that the reactivity dependence on protein density is maintained in this reversal strategy. This simple procedure has proved consistently reliable and is to be recommended as an expedient method where the loss of immunosorbent capacity can be tolerated by the savings of time and effort involved in conventional elution and re-immobilisation.

TABLE 2

COMPARISON OF THE REVERSAL METHOD WITH THE DIRECT METHOD

Averages of 1-ml samples of Sepharose prepared at 25°.

Direct		*Reversed*	
IgG (mg)	*Reactivity (moles/mole)*	*IgG (mg)*	*Reactivity (moles/mole)*
0.9	1.10	1.0	0.57
1.8	0.70	2.0	0.35
3.5	0.60	3.7	0.34
6.8	0.54	7.0	0.29
12.3	0.50	11.7	0.24

3. AUTOMATIC PREPARATIVE INSTRUMENTATION

The requirements of an instrument system dedicated to preparative separations utilising immunosorbents are comparatively simple. They include a means of sequentially applying sample, washing buffer and eluting buffer to the immunosorbent

column, facilities to separately collect the unadsorbed eluent and the desorbed product and provisions for monitoring and controlling these operations. Schematically the system can be presented as shown in Fig. 4. As the process invariably involves repetitive operation, due to the limited capacity of the immunosorbent column, additional means for predetermining the number of cycles to be performed and continuous dialysis of the product are conventionally incorporated into the design.

In using the high flow-rate rapid cycling strategy, referred to earlier, the volume of eluted fractions can be considerable. A modification of the original system

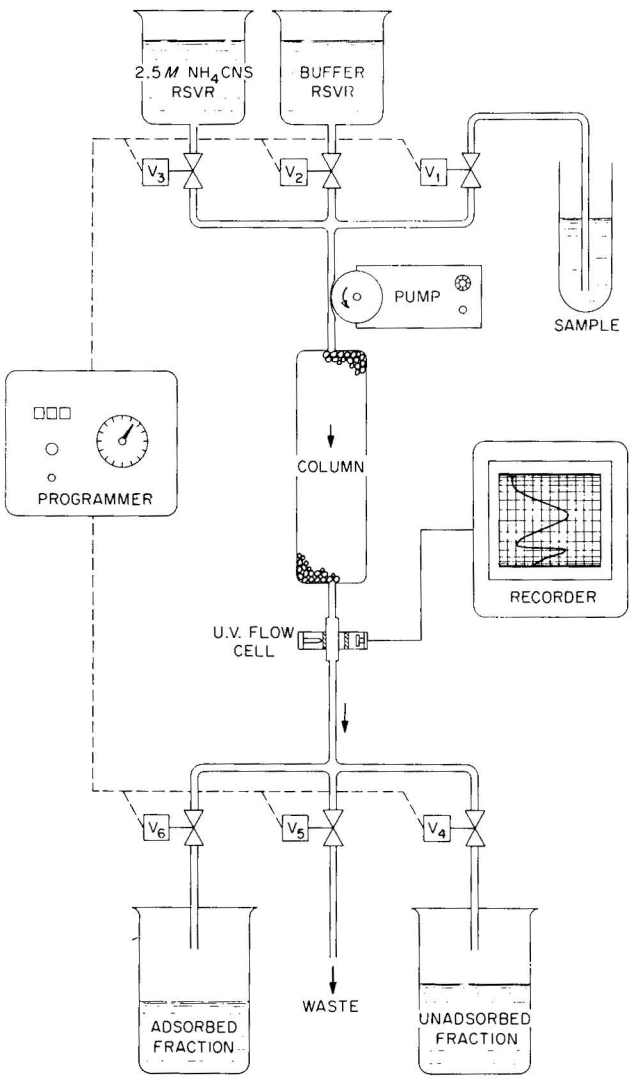

Fig. 4. Schematic representation of preparative affinity chromatographic system.

in which a hollow-fibre cartridge is incorporated into the design, as shown in Fig. 5, largely overcomes this problem. A back-pressure is created in the cartridge by valving the dissociated fraction to a second channel of the peristaltic pump that has a lower flow-rate than the primary column delivery. The resultant internal pressure within the hollow fibres provides continuous dialysis and, more importantly, concentration of the fraction. In practice, a concentration factor of about five is possible without damage to the integrity of the fibres.

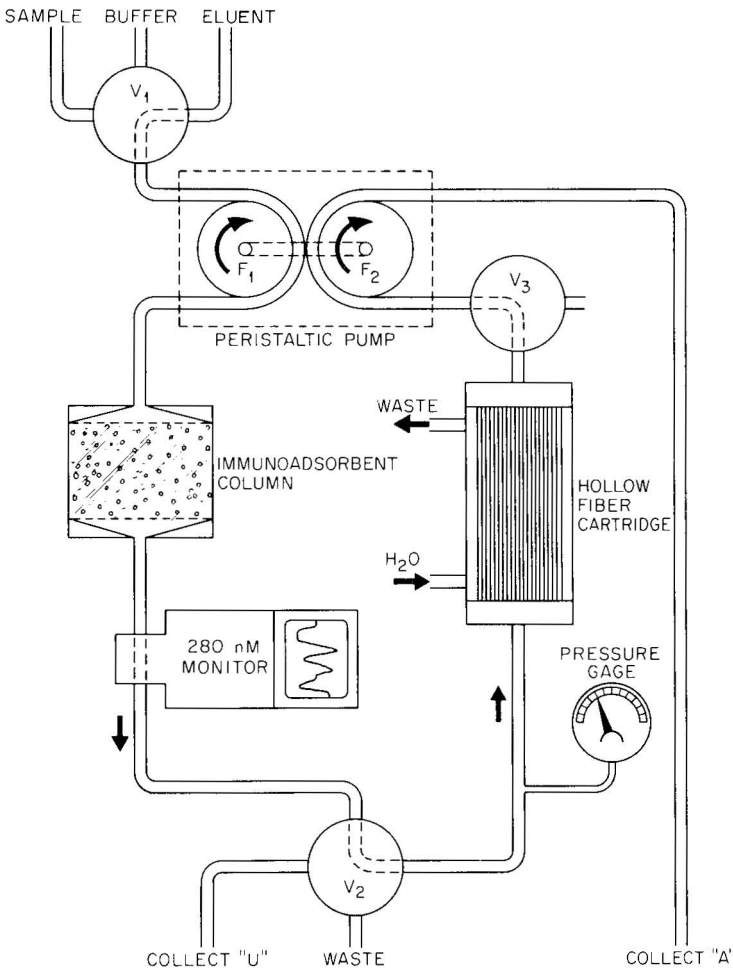

Fig. 5. Schematic diagram of continuous dialysis and concentration preparative system.

The earlier instrument systems, which are described in detail by Anderson *et al.*[8,9], were constructed using cam-activated programmers in conjunction with automatically resetting clocks. An example of one of these earlier models is shown in Fig. 6. Although adequate for long-term routine processing of antigen preparations

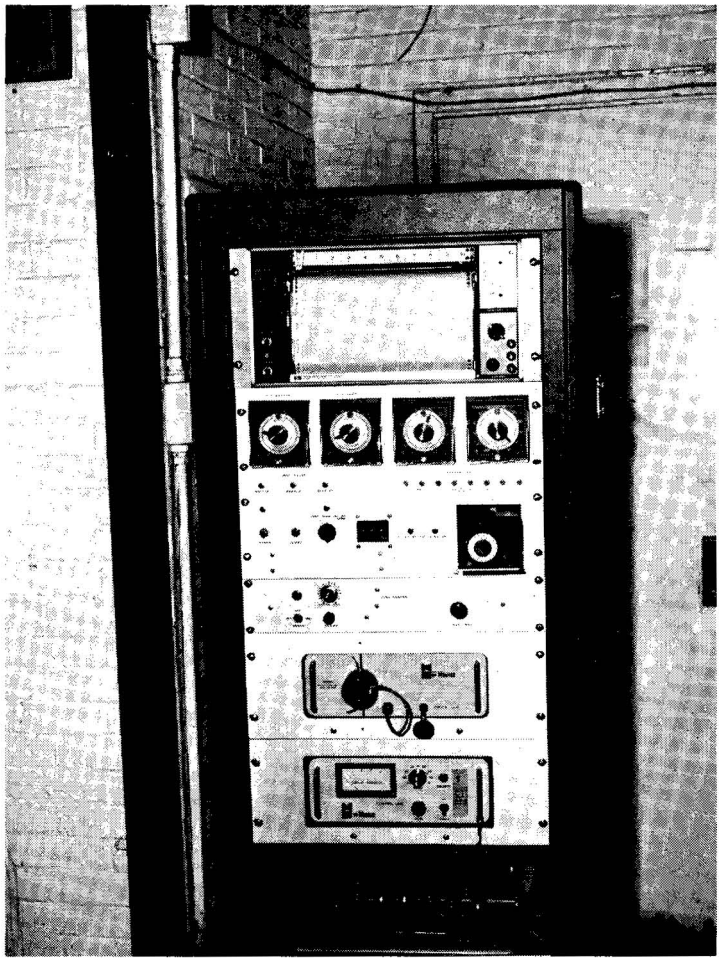

Fig. 6. Early automatic immuno-affinity system.

or antisera, they suffered from the disadvantage of cumbersome programming and restrictions in flexibility for small-scale research applications.

The next generation of automatic immuno-affinity instruments, by now known as "Cyclums", incorporated a more easily adjustable programmer and an increased versatility in operational parameters. A circular-faced timing unit not only provided a visual representation of the total programme but further provided facilities for overlapping functions and for minor timing adjustment during the operation of the system. These latter improvements are essential in optimisation of the protocols in the initial setting-up operation. Several free-standing systems, as illustrated in Fig. 7, were constructed and are in use in a few laboratories in the U.S.A. under collaborative research programmes. A smaller bench-top system was made for the application of low-volume columns, where a higher degree of resolution was necessitated;

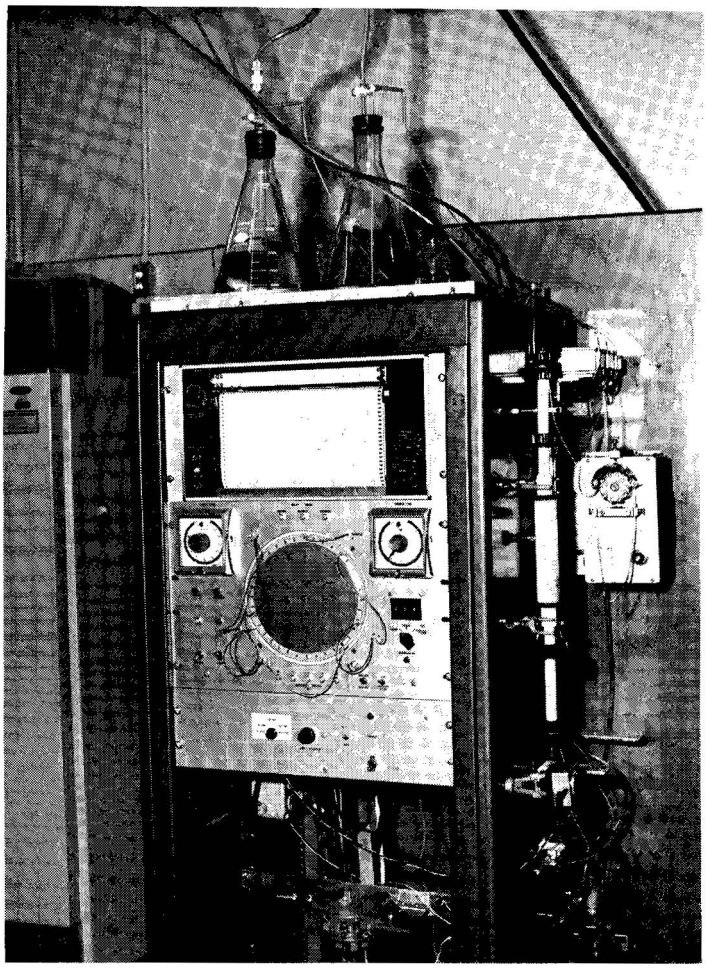

Fig. 7. "Cyclum" automatic immuno-affinity chromatography system with rotary programmer.

the single model constructed is shown in Fig. 8. Finally, in this generation of instruments, a solid-state digital programmer was developed that had the advantage of no mechanically moving components and high reliability in operation. All of these instruments were however dedicated systems, their application being restricted to conventional repetitive immunosorbent separation procedures.

Our increasing awareness and technical experience in exploiting immunosorbents for preparative applications prompted the development of a new concept in chromatographic separation technology, that I now would like to present briefly for the first time in public. This instrumental system, which we have christened the ARK (Automated Repetitive Chromatograph), extends the principle of the immuno-affinity instrument systems into all areas of preparative chromatography.

Basically we have designed an instrument system that can be pre-programmed in "users language" to perform most, if not all, of the procedures required in any

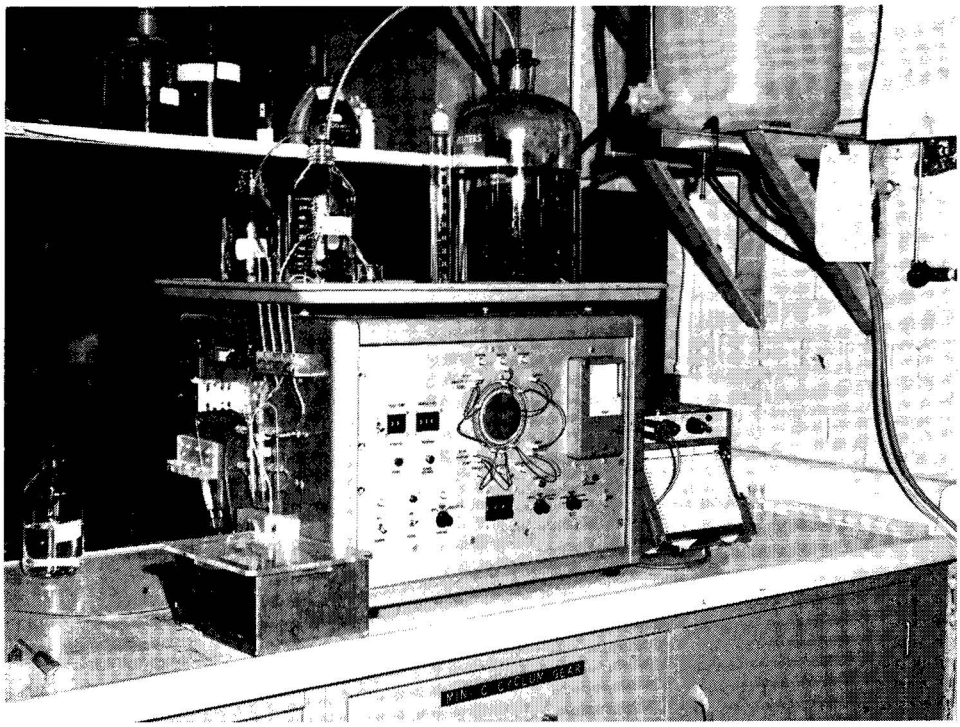

Fig. 8. Bench-top miniaturised automatic immuno-affinity chromatography system.

chromatographic separation in which the separation media can be considered reusable or regenerable. To obviate the dependence of column dimensions and capacity on sample size or concentration, we have adopted the principle of using a standard column and repeating the separation until the total sample has been processed.

Using this standard column, the operating protocol can be optimally predetermined for the required separation and stored as a "machine language" programme for later reactivation and practical use. The encoded programme contains all essential operating and system parameters, such as fluidic connections, timing intervals, collection requirements and general housekeeping duties of the instrument. Safety features, such as fluid detectors and synchronisation signals, ensure reliable and unattended automatic operation with preservation of valuable samples and products under unforeseen circumstances. The only variable remaining to the investigator in reactivating a separation, is to adjust the sample concentration, or the application volume, to a value within predetermined optimal limits.

The basic design of an instrument to give this operational versatility can be conveniently divided into two elements, the hardware and the software facilities. In using these terms, we intentionally are plagiarising the language usually associated with computer design and operation, the analogy with this technology being convenient as well as appropriate. In principle, the hardware of the ARK is fairly conventional and is schematically shown in Fig. 9. The input and output versatility of

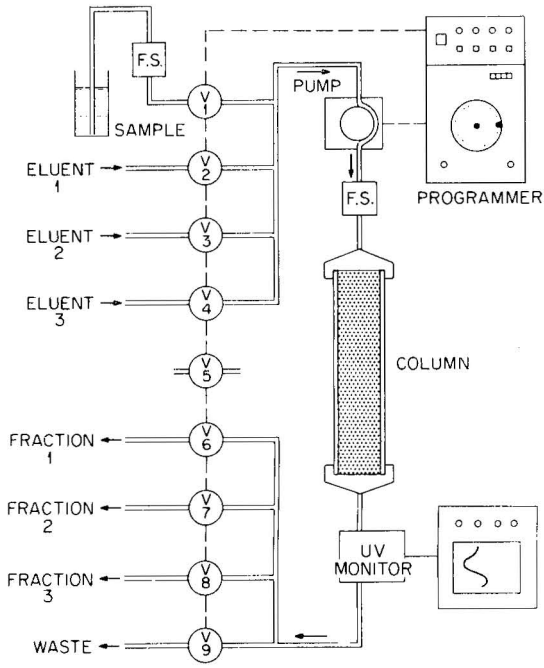

Fig. 9. Schematic representation of Automatic Repetitive Chromatography (ARK) system.

```
   ARK PROGRAM:   SIMPLE IMMUNOSORBENT SEPARATION

LINE    TIME                  INSTRUCTION
0010     T1     IN: SAMPLE;   OUT: WASTE;
0020     T2     IN: BUFFER;   OUT: WASTE;
0030     T3     IN: BUFFER;   OUT: COLLECT U;
0040     T4     IN: SCN;      OUT: COLLECT U;
0050     T5     IN: SCN;      OUT: DIALYZE A, COLLECT A;
0060     T6     IN: BUFFER;   OUT: DIALYZE A, COLLECT A;
0070     T7     IN: BUFFER;   OUT: WASTE;
0080     T8     IF: FS1 = 1, RESET TO 0010; ELSE: STOP;
```

Fig. 10. User language program for simple immunosorbent repetitive separation.

the column is determined by an array of fluid switches under the control of the software programmer. Fluidic sensors (F.S.), consisting of small encapsulated light sources and detectors that signal the presence of air in fluid lines, are strategically placed in the circuit. These devices continuously monitor significant fluid lines and inform the programmer when the sample has been completely processed. Conventional UV monitoring facilities and a multi-channel peristaltic pump complete the basic instrumental system.

The relationship between the hardware components of the system and the software programming is best described by using a specific example of a separation. The user language programme for a simple immunosorbent repetitive separation,

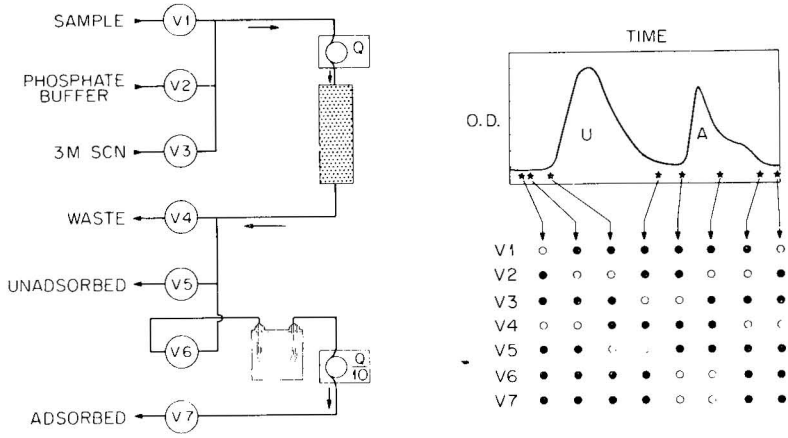

Fig. 11. Fluidic circuit and program logic for simple immunosorbent separation on ARK.

with dialysis and concentration of the desorbed product, is shown in Fig. 10. Here the logic of the required functions is defined in terms directly relevant to the separation. The timing sequence (T1–T8) is to be determined empirically and is dependent upon the capacity of the immunosorbent, the column flow-rate, and the degree of purity required in the product. Once these times have been determined, however, they become an integral part of the software programme and are thus retained for future re-use of the column.

In translating these requirements into an operational programme, a fluid circuit diagram and a machine language listing is required. This is illustrated in Fig. 11 for the simple immunosorbent separation including dialysis and concentration. The inputs to the column and the collection requirements are now defined as practical

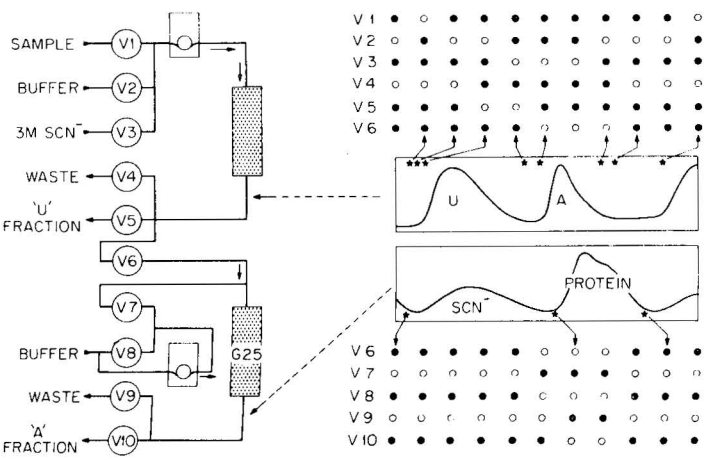

Fig. 12. Fluidic circuit and program logic of immunosorbent separation with concurrent gel filtration of desorbed product.

connections to the array of fluid switches. The sequential operation of this array is reduced to a matrix of switching instructions to be activated at the time intervals stored in the programmer. This matrix is shown at the right of the figure related to the output of the column; the input instructions are of course anticipatory and preceed the output signal by a constant interval dependent upon the void volume of the column. The programmer controls not only the fluid switch array but is further programmed, by means of subroutines, to respond to instructions that encompass safety logic, recycling conditions and other specific requirements.

One of the major advantages of the system is the capability of programming two or more separation columns in serial operation. This is illustrated in Fig. 12 which shows the circuit and logic for the immunosorbent separation with concurrent

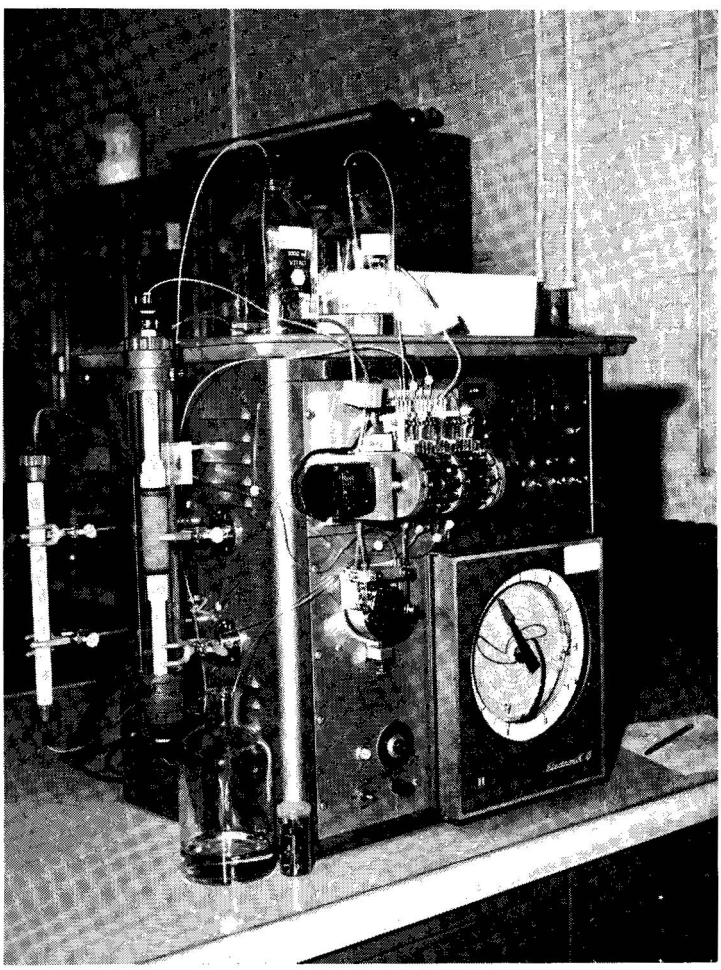

Fig. 13. Prototype Automatic Repetitive Chromatograph.

gel filtration of the desorbed product to remove chaotrope ions. Although the fluid circuit and the operational programme appear complex, in practice it is only marginally more difficult to optimise operating conditions compared with a single column separation. In this context, it is advantageous to tailor the secondary column to suit the elution characteristics of the primary separation and thus retain efficient synchronisation and overlap of functions.

The prototype instrument that we have constructed employing these concepts is shown in Fig. 13. The repetitive separation being performed is that of an immunosorbent column in conjunction with a Sephadex G25 gel filtration, as detailed in Fig. 12. The two columns can be seen on the left, and a multi-way programmable valve and a circular chart recorder on the front of the instrument. Above the circular recorder, which is additionally adapted to be a time programmer, is the main control panel and on its left the peristaltic pump. The output of the immunosorbent column is connected through the minaturised UV monitor, the signal of which is being recorded on the circular chart recorder. The variable rotation time of this chart is presettable and, by means of a peripherally placed sequence of holes, photometrically provide the required timing signals.

The multi-way programmable fluidic valve that we have used in this prototype, is shown in more detail in Fig. 14. By means of lever mechanisms, fluid lines

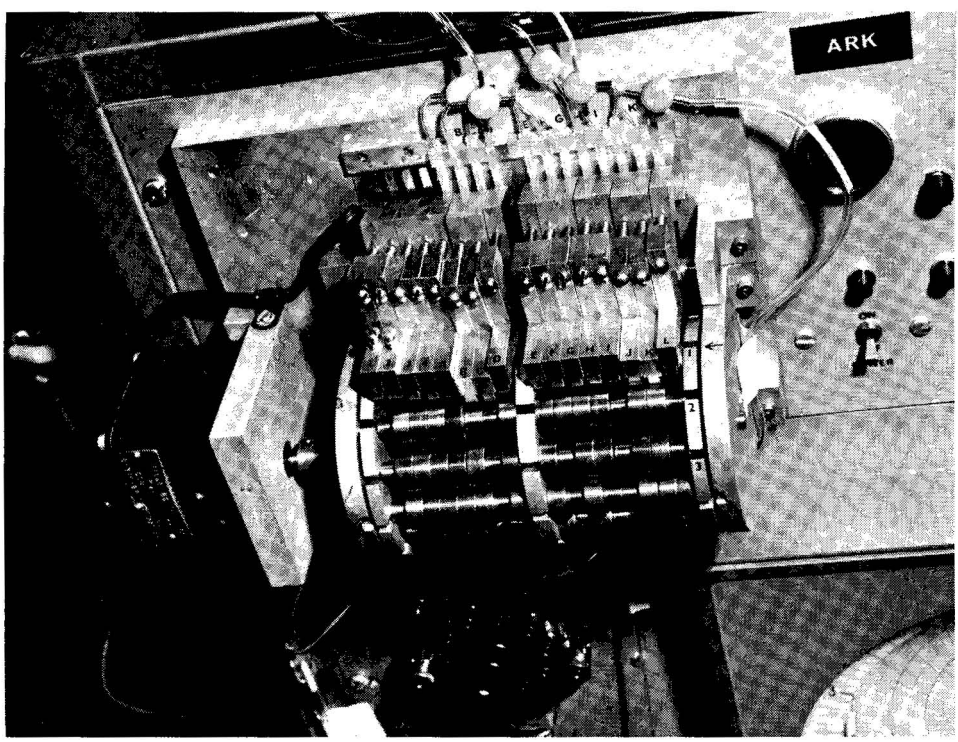

Fig. 14. Multi-way programmable fluidic valve.

are opened or closed by prior insertion of small- or large-diameter rollers on the drum shafts. The order of these rollers along a particular shaft is dictated by the software program discussed earlier, the shafts corresponding to each operational sequence. Upon receipt of a timing signal from the programmer, the drum indexes one position thus opening or closing the fluid lines according to the pre-programmed sequence. This valve has twelve fluid channels with a further four micro-switched electrical channels and a repetitive indexing sequence of twelve operating modes. We have found this degree of versatility to be adequate for all of the chromatographic separations that we have attempted. It is obvious that there are several alternative means of improving the hardware components of this prototype and we are currently investigating the application of microprocessor technology to the design of a fully automated and readily programmable instrument.

My colleague, Mr. David E. Levy, has successfully programmed the following preparative separations on this prototype in addition to the immunosorbent protocols previously outlined. Gel filtration for both simple desalting and more complex fractionation of protein solutions; separation of IgG on a routine basis using QAE-Sephadex and ethylenediamine buffers for elution with acetate buffer for regeneration; isolation, with concurrent concentration in a secondary column of specific proteins using DEAE-agarose and a step-wise buffer elution sequence; fractionation of ribonucleic acids on a hydroxyapatite column using a regenerable gradient subroutine. In addition to these established separations, application of the instrument to specific requirements in immunosubtraction and depletion studies have further justified the development of the system.

In conclusion, I would like to introduce briefly two future applications of immunosorbents that we are now studying. The idiosyncratic response of immunised animals is a constant source of irritation to an immunochemist dependent upon this biological system for his reagents. To obviate this dependence, we have initiated a programme with the objective of fractionating the spectrum of antibodies produced in immunisation into populations of defined affinity and, to a certain extent, specificity. Theoretical explorations have outlined the parameters necessary for this separation and we are currently designing a practical system based on these feasibility studies. Briefly it appears that a continuous counter-current system using antigen immunosorbents will provide the required preparative capability, although a true chromatographic approach has not been entirely ruled out. The justification for this effort is related to the improved sensitivity of radioimmunoassays promised by the defined high-affinity fractions that result from the separation.

The other future application of immunosorbents is related to *ex vivo* immunodepletion of specific antibodies from immunised animals and to a potential therapeutic strategy for the amelioration of certain human diseases. These applications are made possible by a blood cell separator program within our laboratory. The major objective of this latter program has been to improve granulocyte separation capability for replacement therapy of cancer patients undergoing chemotherapy. The blood cell separator however, also provides a continuous cell-free plasma stream into which a sterile immunosorbent containing cartridge can be incorporated for continuous specific immunodepletion purposes. In therapeutic applications the cartridge would remove from the circulating plasma stream specific immune blocking factors such as antigens, immune complexes and idiotypic antibodies that are implicated in the

etiology of neoplasia. Removal of these blocking factors will allow the natural immune defence system to again become effective.

In addition to the human blood cell separator, we have developed a low-volume rotor for the continuous separation of plasma from small animals. Incorporation of an antigen immunosorbent cartridge in this *ex vivo* loop will allow continuous removal of specific antibodies from the animal and thus provide a theoretical increase of an order of magnitude in yield over weekly bleedings. The use of the ARK in this future application of immunosorbents will allow a continuous recycling of two cartridges, thus reducing the total amount of antigen immunosorbent required in the strategy. More important than this potential of an increased yield of antibody is perhaps the biological question as to the immunological response of the animal to this specific depletion with regard to affinity and specificity of the regenerated antibody population.

Seeking answers to questions such as this provides the stimulus in our continuing endeavours to exploit fully the potential application of immunosorbents. An innovative approach, based originally upon a touch of Swedish serendipity, will hopefully provide the answer and hence the reward. It is in this context that the invitation to attend this Symposium and its opportunity to share with you a few of the answers that we have already found, is, in itself, an honour and an ample reward.

4. SUMMARY

Studies of the physical and chemical characteristics of immunosorbents have allowed operational improvements to be made in their application to preparative separation of pure antigens and monospecific antibodies. The loss of immunochemical reactivity with increasing protein substitution and the limitations imposed by the porous nature of many supports, have been significantly overcome by improved techniques and operational protocols. The development and operation of dedicated immuno-affinity chromatographic systems designed for routine preparative applications are described. A new automatic repetitive chromatography system, generally applicable to a wide spectrum of routine biochemical preparative separations, is presented for the first time.

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SOME CURRENT AND POTENTIAL USES OF MAGNETIC FIELDS IN ELECTROKINETIC SEPARATIONS

ALEXANDER KOLIN

Institute of Molecular Biology, University of California, Los Angeles, Calif. (U.S.A.)

CONTENTS

1. IDEAS WHICH LED TO USES OF MAGNETIC FIELDS IN ELECTROKINETIC SEPARATIONS

I always find it instructive to attempt to reconstruct the sequence of ideas which lead to a given scientific development. In retrospect it seems to me that there is a thread of continuity which leads from my work on electrophoresis and electromagnetophoresis back to a seemingly completely unrelated development, namely, the electromagnetic method for measuring blood flow.

In 1935[1,2] I set myself the goal of developing a method for measuring the flow of blood in an artery without opening or penetrating its wall. The idea which led to the solution came to me as an analogy to the Hall effect. If we send a current (represented by vector $\vec{V} = \vec{J}$ in Fig. 1) through a cylindrical conductor, such as a wire (C in Fig. 1), while maintaining a magnetic field ($\vec{B}$) at right angles to the current, an e.m.f. (the Hall voltage) can be detected by the meter MS across a cylinder diameter (between the electrodes E_1 and E_2) perpendicular to the magnetic field. If we imagine

positive as well as negative charge carriers to be present in the conductor they will be moved in opposite directions along the x-axis direction by the current source. The Lorentz force exerted upon these moving charges at right angles to the magnetic field will, however, be in the same direction for the positive as well as negative charges because their signs as well as directions of motion are mutually opposite. Thus, if the charge densities of both charge carriers were the same, there would be no potential difference across a cylinder diameter in the direction of the Lorentz force (z-axis) since opposite charges are moved in the same direction. The Hall effect is of great importance to determine the sign and space-density of electrons or holes in semiconductors from the sign and magnitude of the Hall coefficient $R = 1/nec$, where n is the space density of the electrons, e the electron charge and c the velocity of light. In electrolytes, however, freely moving ions of opposite sign are present in equal concentration and the Hall voltage detected between electrodes E_1 and E_2 is zero.

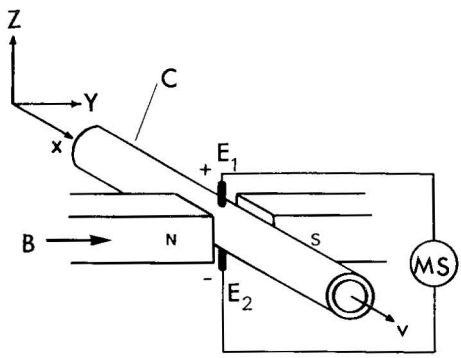

Fig. 1. Cylinder C between magnet poles N and S. $\vec{B}$, magnetic field vector; E_1 and E_2, electrodes; $\vec{V}$, vector specified in text; MS, instrument specified in text.

It occurred to me that this latter situation will be drastically changed if, instead of moving the charge carriers in opposite directions by an electric field, we would move them in the same direction by fluid flow through a pipe C in Fig. 1 across a magnetic field. In this case the Lorentz forces will act in opposite directions on the charge carriers of opposite sign and we will obtain a charge separation and an electric field opposing the Lorentz force. The charge separation will proceed until the electric field produced by it balances the Lorentz field $\vec{E}$ seen by the charges moving with velocity $\vec{v}$ across the magnetic field $\vec{B}$ ($\vec{E} = [\vec{v} \times \vec{B}]$). Our meter MS will then detect a potential difference between the electrodes E_1 and E_2 contacting the fluid which will be proportional to the fluid velocity $\vec{v}$ and we would have an electromagnetic flow meter before us. Material properties like electrical conductivity or space density of charge carriers do not appear in our equations so that the reading of the meter MS will be the same for all fluid conductors in a given field $\vec{B}$ at a given fluid velocity $\vec{v}$. What we have just described is nothing other than the process of electromagnetic induction in a moving fluid. It is in terms of this more familiar concept that this idea has been described in the literature[1,2].

After demonstrating the above effect in electrolytes and using it to measure blood flow with electrodes making contact with the outside of an artery wall, it occurred to me that this effect could be inverted. By replacing the meter MS with a current source and sending an electric current through the electrolyte between the electrodes E_1 and E_2 in the transverse magnetic field $\vec{B}$, a force was exerted upon the electrolyte in the direction of the x-axis and the fluid was set in motion in the direction of the vector $\vec{V}$ in Fig. 1. The rate of pumping was so embarrassingly small and the experiment looked so obvious that I did not take the trouble to publish it. This magnetohydrodynamic pumping action has, however, found in recent times many important uses and I have utilized it in an electrophoretic application that will be described in the last section of this paper.

2. ELECTROMAGNETOPHORESIS

The above experience undoubtedly triggered the following question which occurred to me in the midst of a lecture to medical students. I was describing how the Lorentz force exerted upon ions in an electrolyte-filled rubber tube carrying a current across a magnetic field is transmitted by the ions to the tube. I suddenly visualized a sphere suspended in the tube and asked myself if an object surrounded by the current-carrying electrolyte under these conditions would experience a force like the tube wall. A little thought after the lecture led me to the conclusion that a dielectric sphere would indeed experience a force but that the force would be opposite to the force exerted upon the wall of the rubber tube. It soon became clear that the force would be zero upon a suspended object of an electrical conductivity matching that of the surrounding electrolyte. It would have the same direction as the force upon a tube for a body of higher electric conductivity than the electrolyte and would point in opposite direction for a body of lesser conductivity than the electrolyte. It soon became clear that this was a phenomenon having more resemblance to gravitational sedimentation than to electrophoresis and that its dependence on the relative conductivities of the suspended object and the ambient electrolyte was analogous to the phenomena of flotation governed by Archimedes' principle. I called this effect electromagnetophoresis[3,4]. Even an approximate theory of this phenomenon is rather complex[5,6]. We shall consider it here in a simplified rough approximation.

Fig. 2 shows an apparatus in which this effect can be observed. A Helmholtz coil pair generates a magnetic field $\vec{B}$. A migration cell placed between the coils has the shape of a parallelepiped whose top and bottom surfaces are metallic and serve as electrodes E_1 and E_2, while the remaining walls are made of dielectric material. The current density $\vec{J}$ maintained through the electrolyte between the electrodes is perpendicular to the magnetic field $\vec{B}$. The force density, *i.e.* the force per unit volume exerted upon the electrolyte is

$$\vec{F}^* = [\vec{J} \times \vec{B}] \tag{1}$$

(where $\vec{F}^*$ is in dynes/cm^3, $\vec{J}$ in abamperes/cm^2 and $\vec{B}$ in gauss). This force is analogous to the weight density, the force exerted upon a unit volume in the gravitational field.

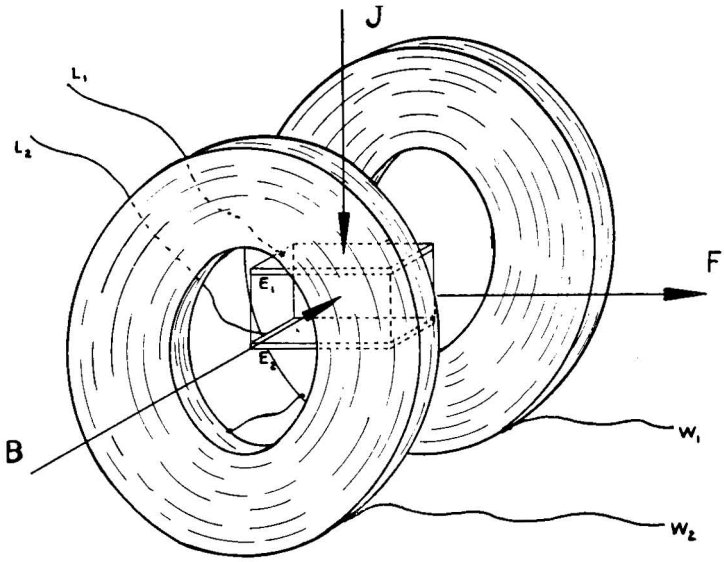

Fig. 2. Arrangement for demonstration of electromagnetophoresis. A migration cell with electrodes E_1 and E_2 is placed between two Helmholtz coils. $\vec{B}$, magnetic field vector; $\vec{J}$, current density vector; $\vec{F}$, electromagnetic force; w_1, w_2, coil lead wires; L_1, L_2, electrode leads. (From A. Kolin, *Proc. 1st Nat. Biophys. Conf.*, Yale University Press, 1959.)

We shall consider the horizontal electromagnetic force field as a quasi-gravitational field. While the gravitational force generates a pressure gradient in the fluid so that the pressure increases in the downward direction, the horizontal quasi-gravitational force in Fig. 2 similarly generates a horizontal pressure gradient with the hydrostatic pressure increasing in the direction of the $\vec{F}$ vector. For simplicity, we shall imagine that our experiment is performed in a freely falling system, such as a satellite in orbit where there are no gravitational manifestations, so that the electromagnetic force field is the only field to be considered.

As a consequence of the downward increase in pressure in a gravitationally induced fluid pressure gradient, the hydrostatic pressure is highest at the lowest points of a submerged object and lowest at the highest points. If we calculate the resultant pressure force exerted upon the surface of the submerged object by integration over its entire surface, we obtain a force pointing in the direction of diminishing pressure (*i.e.* opposite to the force of gravity) of a magnitude equal to the weight of the ambient fluid displaced by the submerged body. This is, of course, the well known force of buoyancy. Similarly, we can perform an analogous calculation in our electromagnetic force field generated as shown in Fig. 2. The result is a surface force $\vec{F}_s$ experienced by each volume element of the electrolyte as a result of the ambient fluid pressure gradient. The direction of this force is, as in the gravitational case, opposite to the force $\vec{F}$ of Fig. 2. In fact, this force is analogous to the force of buoyancy in the gravitational field. It will be convenient for us to adopt a similar terminology, referring to this surface force as "electromagnetic buoyancy" and to the opposite force exerted upon the interior of the volume element as "electromagnetic gravity".

We can now understand why a non-conducting submerged object like a

dielectric sphere will experience a force in the electrolyte-filled cell of Fig. 2 in spite of the fact that it passes no current, so that no electromagnetic forces can be exerted upon its interior. What it suffers is the surface force of electromagnetic buoyancy which is opposite to the direction of the force which generates the pressure gradient in the ambient fluid. If the submerged object is a conductor, it passes a current and its interior experiences a force, "electromagnetic gravity" (EMG) (in the direction of force $\vec{F}$ of Fig. 2), in addition to the opposite surface force of "electromagnetic buoyancy" (EMB). The net force upon the body is the sum of EMB and EMG. In the case of an object whose electrical conductivity σ' is identical with the conductivity σ'' of the surrounding fluid the forces of EMG and EMB are equal and opposite and the resultant force is zero in analogy to isopyknic equilibrium in the gravitational field.

The quantity which is analogous to the density in our gravitational analogy is the electrical conductivity σ. Fig. 3 shows how the value σ' of the conductivity of a body submerged in a fluid of conductivity σ'' affects the current density in the body. The sphere of $\sigma' = \sigma''$ in Fig. 3A is "electrically transparent". The current traverses it without change in current density. Since the current density $\vec{j}$ in the body interior determines the value of the volume force density $\vec{F} = [\vec{j} \times \vec{B}]$, we see that EMG + EMB = 0 in this case and we will not expect a particle of this electrical conductivity to migrate in the given electrolyte.

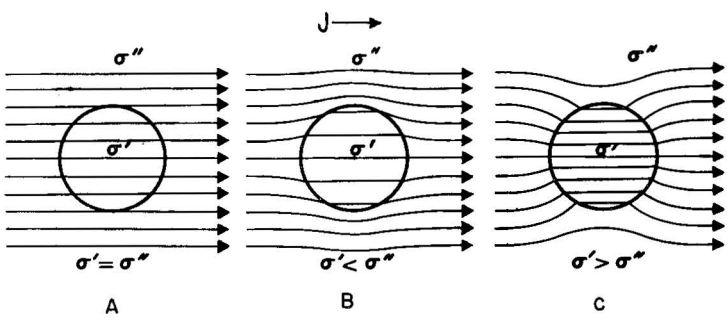

Fig. 3. Refraction of electric current lines. Distribution of current density in a sphere of conductivity σ' in a surrounding fluid of conductivity σ''. (A) $\sigma' = \sigma''$; (B) $\sigma' < \sigma''$; (C) $\sigma' > \sigma''$. In all cases the current density inside the sphere is uniform. It is the same as in the surrounding fluid in A, diminished in B, and increased in C. [From A. Klin, *Science*, 117 (1953) 134.]

On the other hand, we see in Figs. 3B and 3C how the current distribution is changed by suspended spheres when $\sigma' \neq \sigma''$. Such changes in current distribution with concomitant refraction of the electrical current lines at the interface have been calculated by Maxwell[7]. We see in Fig. 3B how the current tends to flow around a poor conductor ($\sigma' < \sigma''$) in which the current density is less than at "infinite" distance in the ambient fluid. Consequently EMG < EMB in this case. The surface force predominates over the volume force and the sphere moves opposite to the direction of the $\vec{F}$ vector in Fig. 2. On the other hand, the superior conductor ($\sigma' > \sigma''$) tends to concentrate the current lines in its interior, thus increasing the current

density in it above the value of $\vec{J}$ in regions of unperturbed current density. Such a more highly conductive object will act similarly to a denser body suspended in a fluid in the field of gravity. It will move in the direction of the force exerted upon the fluid volume as a whole (direction of $\vec{F}$ in Fig. 2). Thus two species of particles of conductivities σ_a' below and σ_b' above the conductivity σ'' of the ambient fluid would move in opposite directions and be easily separated.

Calculations[3-5] valid for particles of cellular dimensions (radius $a \gtrsim 10^{-2}$ cm) yield the following equations from which the forces (F) upon and migration velocities (v) of spherical particles of radius a and of conductivity σ' suspended in a fluid of conductivity σ'' and viscosity η can be calculated:

$$F = 2\pi a^3 [J \times B] \left(\frac{\sigma' - \sigma''}{\sigma' + 2\sigma''}\right) \tag{2}$$

$$v = JB \left(\frac{\sigma' - \sigma''}{\sigma' + 2\sigma''}\right) \frac{a^2}{3\eta} \tag{3}$$

We see from both equations that the force and velocity vanish for $\sigma' = \sigma''$ and that their signs reverse as the value of σ' increases from $\sigma' < \sigma''$ to $\sigma' > \sigma''$.

A numerical example will give an idea of the order of magnitude of this effect. We assume non-conducting particles ($\sigma' = 0$) of radius $a = 10^2\,\mu\text{m} = 10^{-2}$ cm, $J = 0.1\ A/\text{cm}^2$, $B = 10^4$ gauss, $\eta = 10^{-2}$ poise. Eqn. 3 yields for this case $v \approx 1.67$ cm/sec or 83.5 diameters/sec. Superconducting magnets of much higher intensity than assumed here are currently available permitting to obtain much greater migration speeds at higher magnetic fields.

In addition to the capability of electromagnetophoresis to discriminate between particles of different radius (a) and electrical conductivity (σ') as suggested by eqn. 3, it offers the possibility to separate particles on the basis of differences in shape as suggested by Fig. 4 (refs. 3, 4). We shall consider the sphere of Fig. 3C ($\sigma' > \sigma''$) and imagine it deformed (without change in volume) into a prolate spheroid seen in Fig. 4A. The concentration of the current in the submerged body is much greater for

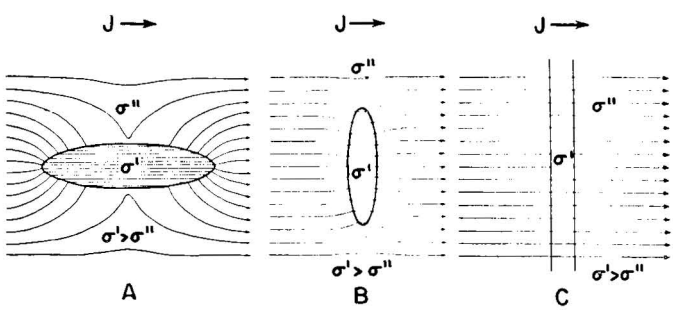

Fig. 4. Effect of shape on current density j inside a body of conductivity σ' immersed in a fluid of conductivity $\sigma'' < \sigma'$. (A) Prolate spheroid: current lines are strongly refracted at interface and internal current density is uniform and greatly increased as compared to unperturbed current field. (B) Oblate spheroid: internal current density is only slightly increased. (C) Infinitely wide disk (lateral view): no refraction of current lines; internal and external current densities are equal. [From A. Kolin, *Science*, 117 (1953) 134.]

this shape and orientation than for a sphere of equal conductivity[3,4] as depicted in Fig. 4A. In view of the greatly increased internal current density j', the submerged body will act like a body of greatly increased electromagnetic weight density. Thus, suspended particles differing but little in electrical conductivity and volume could be separated on the basis of differences in shape which would produce a difference in electromagnetic weight densities.

A deformation of our sphere into an oblate spheroid as shown in Fig. 4B would have a different effect. The internal current density j' in this body will be less than for a sphere of equal volume and conductivity σ'. The oblate spheroid will thus behave like a body of lesser electromagnetic weight density than the sphere of equal volume and conductivity and will exhibit a much smaller electromagnetic weight density than an equiconductive prolate spheroid of equal volume[3,4]. The plate of conductivity σ' can be considered as a limiting case of a prolate spheroid whose thickness is vanishingly small as compared to its lateral dimensions. There is no refraction of current lines in this case depicted in Fig. 4C for the shown orientation. The current density in the plate is the same as outside. The forces of EMB and EMG thus cancel each other and the conductive plate placed perpendicular to the current experiences no force.

Table I illustrates effects of orientation of a cylinder upon the current density in it, and thus upon the manifested electromagnetic weight density[4,5].

TABLE I

ORIENTATION-DEPENDENCE OF FORCE UPON A CYLINDER

Body	*Orientation**	*Force density F*	*Special conditions*
Sphere		$\frac{3}{2}[JB]\left(\frac{\sigma'' - \sigma'}{2\sigma'' + \sigma'}\right)$	
Cylinder	$\vec{B}$	0	
Cylinder	$\vec{J}$	$[JB]\left(\frac{\sigma'' - \sigma'}{\sigma''}\right)$	$\frac{L}{a} \gg \frac{\sigma'}{\sigma''}$
Cylinder	$\vec{F}$	$[JB]\left(\frac{\sigma'' - \sigma'}{\sigma'' + \sigma'}\right)$	

* Cylinder axis parallel to vector indicated.

We have treated electromagnetophoresis so far, as if it were produced by a combination of a constant magnetic field with a perpendicular direct current. Actually, this is not necessarily the most favorable case because of electrolysis at the electrodes. By periodically reversing the magnetic field in phase with reversals of the electric current we exert a unidirectional force upon suspended particles. The use of an alternating current has another aspect of interest. A biological cell is not an homogeneous conductor. The cell interior is surrounded by a cell membrane which can be, electrically, roughly approximated by an equivalent circuit of a resistance in series with a parallel combination of resistance and capacitance. The impedance of the

cell membrane is thus frequency-dependent and so will be the density of the electric current passing through the cell. We thus gain a further parameter, the frequency characteristics of the cell membrane impedance, according to which biological cells may be separated by electromagnetophoresis.

In our gravitational analogy we consider electromagnetophoretic migration as an analog to gravitational sedimentation. The analogy can also be extended to centrifugation. We can produce a radial electromagnetic force field in which particles differing from the suspension fluid in the value of the conductivity σ will migrate either toward a center of convergence or away from it, just as they would in a centrifuge on the basis of density differences[8].

Fig. 5 shows the simplest kind of electromagnet, a wire W carrying an electric current which generates a solenoidal magnetic field (H). (Actually a water-cooled copper tube passing an a.c. current of 3,500 A derived from a step-down transformer was used). The wire passes through a cylindrical migration cell whose flat walls serve as electrodes, E_1 and E_2. The current density $\vec{J}$ between the electrodes is parallel to and in phase with the current $\vec{I}$ that is passed by the wire. The circular magnetic field lines are thus perpendicular to $\vec{J}$ and the Lorentz force $\vec{F}$, being perpendicular to $\vec{J}$ and $\vec{H}$, is radial. Particles suspended in a fluid of conductivity σ'' will thus exhibit centrifugal or centripetal migration depending on the sign of $\sigma' - \sigma''$. To obtain centrifugation without rotational motion one need not use a circular cell as shown in Fig. 5. Its cross-section may be a semicircle or a sector of less than 180° angular opening could be used so that the wire W would not have to pass through the cell interior.

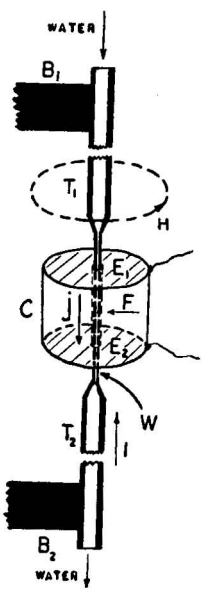

Fig. 5. Electromagnetophoretic non-rotational centrifuge. W, Conductor generating a solenoidal magnetic field; C, migration cell; E_1 and E_2, electrodes; J, current density in cell C; I, current in conductor W; T_1 and T_2, wide copper tubes linked to hollow conductor W; B_1 and B_2, current leads. [From A. Kolin, *J. Appl. Physics*, 25 (1954) 1065.]

3. ELECTROMAGNETOPHORESIS AND CURRENT INTEREST IN CELL SEPARATION METHODOLOGY

While reasonably satisfactory electrophoretic methods are currently available for cell electrophoresis[9–12], there are some serious limitations which may require development of new separation methods. To maintain a high electric field intensity and electrophoretic mobility of cells it is necessary to use buffers of low ionic strength and electric conductivity. Non-electrolytes such as sucrose must be added to avoid osmotic damage to the cells. The cells must remain for an appreciable period of time in a less than optimal environment differing considerably from their natural habitat. An ideal separation method would be one permitting, for instance, mammalian cells to remain in an environment like blood serum during the separation process.

Another drawback of electrophoresis as a method of cell separation is the usually relatively small percent difference in electrophoretic mobilities between the cell species which one is interested in separating. A separation method based on a physical parameter that varies more widely from one cell species to another would, of course, be preferable.

It appears that electromagnetophoresis holds out the promise of fulfilling the above desiderata. Unlike electrophoresis, electromagnetophoresis is carried out most effectively in a high-conductivity fluid. Blood serum would be an ideal suspension medium for cell-electromagnetophoresis. This would greatly reduce the problem of power dissipation in the cell suspension by the current. Physiological saline would also be a suitable suspension medium and the use of materials such as sucrose for achievement of osmotic balance would be unnecessary.

Of even greater importance is the fact that biological cells may differ by several orders of magnitude in values of their electric conductivities[13]. Electromagnetophoresis may thus provide a tool of exceptionally high resolution for cell separations. An added factor of interest is that the entry of the current into a biological cell will be determined by the permeability of the cell membrane to ions. There may thus be a prospect of cell separation on the basis of differences in ionic cell membrane permeabilities.

Another prospective advantage is the possibility of using alternating currents which eliminates electrolysis and greatly simplifies the design of the separation cell.

It may also be of interest to compare the electrophoretic velocities in contemporary "free-flow" electrophoresis apparatus with migration speeds achievable by electromagnetophoresis. A human erythrocyte of 25 TU electrophoretic mobility [1 Tiselius Unit (TU) $= 10^{-5}$ cm sec^{-1}/V cm^{-1} (ref. 14)] will move in the field of 100 V/cm in a free-flow or endless belt electrophoresis apparatus with a speed of 0.025 cm/sec. This speed is to be compared with electromagnetophoretic (EMP) speeds calculated from eqn. 3 for a normal laboratory electromagnet of 25 kilogauss and a superconducting magnet of 100 kilogauss. We choose round figures for radii of two types of cells: $a_1 = 5$ μm and $a_2 = 10$ μm which are in the order of magnitude of erythrocyte and lymphocyte dimensions, respectively. The calculated velocity values v are displayed in Table II. We see that the EMP-speed of the smaller cell is a little less than half of the above mentioned electrophoretic speed of an erythrocyte at the weaker magnetic field. However, the speed of the larger cell (large lymphocyte) is four times as great and much greater than the electrophoretic erythrocyte velocity.

The velocity difference between the two types of cells is large as compared to velocity differences normally encountered between such cells in electrophoresis. At the larger magnetic field intensity (100 kilogauss) both types of cells by far surpass the cited electrophoretic migration velocity of the erythrocyte. In fact, the larger cell migrates more than six times as fast and the velocity difference between the two cell types at 100 kilogauss is 300% larger than the slower cell velocity. In addition to the anticipated resolving power on the basis of cell size differences we can expect appreciable resolution on the basis of differences in electrical cell conductivities and ionic permeabilities of cell membranes.

TABLE II

MIGRATION SPEED v OF SPHERE OF RADIUS a IN MAGNETIC FIELD B AT CURRENT DENSITY J

a (μm)	B (kilogauss)	J (abamperes/cm^2)	σ' (Ω^{-1} cm^{-1})	η (poise)	v (mm/sec)
5	25	0.1	0	0.01	0.1
10	25	0.1	0	0.01	0.4
5	100	0.1	0	0.01	0.4
10	100	0.1	0	0.01	1.6

There have been very few attempts as yet to use this effect for practical separations[15,16]. Murphy *et al.*[15] described separation of erythrocytes from blood plasma and Kovalczik[16] presented an extensive theoretical analysis and experimental accounts of electromagnetophoresis in porous media. There are probably several reasons for the long delay in utilization of this effect. (1) Electromagnetic convection due to non-uniformity of the magnetic field and of the electrical current density along with thermal convection present formidable experimental difficulties the solution of which will require no less ingenuity than went into the development of electrophoretic methods by Tiselius and those who followed him. (2) Powerful electromagnets, especially superconducting magnets, in the order of 100 kilogauss became commercially available relatively recently and are still rather costly to acquire and to operate. (3) The interest in cell electrophoresis is also of relatively recent date and it is mainly in connection with cell separations that EMP could find applications with present technology.

4. ISOPERICHORIC FOCUSSING EFFECTS: ISOCONDUCTIVITY AND ISOMAGNETIC FOCUSSING; DIGRESSION TO ISOELECTRIC FOCUSSING

In above descriptions we took it for granted that the electrical conductivity of the electrolyte was uniform throughout the EMP cell. This need not be so. We can extend our gravitational analogy by remembering the method of determination of the specific gravity of small and irregularly shaped objects. We generate a density gradient column such that the object finds a level of density equal to its own someplace within the fluid column. The determination of the fluid density at the level where the object resides yields then its density. We could now similarly create a gradient of electrical conductivity in, say, a vertical column in which we could establish a vertical EMP force. If the conductivity σ'' increases in the downward

direction we have an analogy to a stable density gradient column. Suppose the EMP column contains a suspension of cells of electrical conductivity σ'. At a certain level L_0 of the conductivity gradient column the value of the ambient conductivity will be equal to the cell conductivity: $\sigma_0'' = \sigma'$. The EMP force will not affect the cells at this level. Below this level $\sigma' < \sigma''$ and the EMP buoyancy predominates over the EMP gravity moving the cells upward toward the L_0 level. Conversely $\sigma' > \sigma''$ above the L_0 level and the cells are moved downward toward the level where $\sigma' = \sigma''$. Eventually all of the suspended cells will be swept from above and below toward the L_0 level where they will be focused in an equiconductive zone of the column.

This consideration could have led me to the idea of isoelectric focussing, but actually it did not since I was not thinking about electrophoresis at the time I worked on EMP. It was the above mentioned experimental convection difficulties[17,18] which induced me to switch temporarily to the study of electrophoresis in order to seek ideas that could be usefully transferred to electromagnetophoresis. The idea of isoelectric focussing occurred then in the process of reading about the pH dependence of the charge and electrophoretic mobilities of proteins[19–21].

Further thought about the isopyknic, isoconductivity and isoelectric focussing effects led to the insight that they are special cases of a class of effects which one might call isoperichoric focussing effects (from the Greek word "perichoron" = environment)[21]. These effects occur in a gradient of an environmental parameter (*e.g.* density) where forces are exerted upon suspended particles in those regions of the gradient column where the environmental parameter and the corresponding particle parameter differ. These forces vanish in the "isoperichoric zone" where the force-determining particle parameter equals the corresponding environmental parameter. On either side of this equilibrium zone the forces exerted upon the particles point toward this zone of convergence. By inverting the gradient one can reverse these forces so that the particles would tend to flee the isoperichoric zones (but this condition is in most cases unstable).

A predicted isoperichoric effect which may find applications in cell separations is isomagnetic focussing[21]. There are two possibilities: isodiamagnetic and isoparamagnetic focussing. We will briefly qualitatively outline isoparamagnetic focussing. A paramagnetic body in a non-homogeneous magnetic field will experience a force in the direction of increasing field intensity. Faraday discovered, however, that this was only true if the paramagnetic body was in an environment of smaller magnetic permeability. If the magnetic permeability μ'' of the environment surpasses that of the body (μ'), the latter will experience a force away from the region of maximum field strength. Let us imagine now a vertical column filled with a paramagnetic liquid whose magnetic permeability increases in the downward direction. Let us also assume that this fluid column is in a non-homogeneous magnetic field which increases in the downward direction. Each fluid element will thus experience a downward force and a pressure gradient will be established in the liquid column. The force per unit volume will be greater in proportion to the magnetic permeability of the volume element. In view of the existence of grad μ'' with a μ'' value increasing in the downward direction, we have a situation analogous to a stable density gradient in a gravitational field.

A particle of permeability μ' suspended in this permeability gradient column in a non-uniform magnetic field will experience a downward or an upward force

depending on whether it finds itself above or below the isoparamagnetic zone Z_0 where $\mu' = \mu_0''$. As in the analogy of isopyknic focussing in a density gradient, the paramagnetic particles will be eventually swept into the isomagnetic zone Z_0. Particles of a different permeability μ^* will be focussed in a different isomagnetic zone. Analogous focussing could be obtained with diamagnetic particles suspended in a diamagnetic fluid.

5. CONDUCTIVITY-GRADIENT STABILIZATION AGAINST THERMAL CONVECTION

Density gradients proved very useful for establishing concomitant pH gradients[19] and stabilization in isoelectric focussing as well as for generation of electrophoretic mobility spectra[22,23] and found uses in non-electrokinetic separation procedures. A possibility of establishing stabilizing gradients by other means and, especially, of reinforcing the stabilizing action of density gradients by other types of gradients are therefore of interest. It turns out that one can actually produce very steep stabilizing gradients in conductivity gradient columns and add their stabilizing action to that of a density gradient, thus enhancing the latter's effectiveness in suppressing thermal convection[24].

Fig. 6 shows a cell in which we can generate an EMP force $\vec{F}$ by maintaining a current density $\vec{J}$ between electrodes E_1 and E_2 in the presence of a transverse magnetic field $\vec{B}$. If the conductivity σ of the electrolyte increases, for instance in the downward direction, so will the current density $\vec{J}$ maintained by the potential difference V between the electrodes. Thus, the electromagnetic force density $\vec{F} = [\vec{J} \times \vec{B}]$ will increase with the conductivity σ in the direction of grad σ. We are at liberty to determine the direction of the $\vec{F}$ vector by choosing the direction of the current and of the magnetic field. Let us assume that the force $\vec{F}$ points downward (opposite to the direction shown in Fig. 6). We have now an analogue to a stable density gradient in the gravitational field where the weight density $(\vec{dg})$ is replaced by the electromagnetic force density $[\vec{J} \times \vec{B}]$. Since the direction of $\vec{F}$ points in the direction of increasing $[\vec{J} \times \vec{B}]$ values, the gradient is a stable one. If we were to reverse $\vec{J}$ or $\vec{B}$, we would invert the direction of $\vec{F}$ (as depicted in Fig. 6) and an unstable conductivity gradient would result comparable to a density gradient in which the density is increasing in the upward direction. By adding such an electromagnetically stabilized conductivity gradient to a density gradient, we can obtain a combined gradient of far greater stability[24].

The stability as well as destabilization of such a hybrid gravitationally–electromagnetically stabilized gradient can be demonstrated experimentally[24]. Fig. 7 shows four photographs of the transilluminated cell of Fig. 6. The fluid in the cell is filled with a solution in which a density gradient of a urea solution is accompanied by a conductivity gradient due to non-uniformly dissolved NaCl. The concentration of both solutes increases in the downward direction and, hence, both solutes contribute to the generation of a stable density gradient. In photograph A, the stability of the density gradient is enhanced by turning on a current of 0.5 A per cm^2 of electrode area. The intensity of the transverse magnetic field is 6,000 gauss. (Both, the current

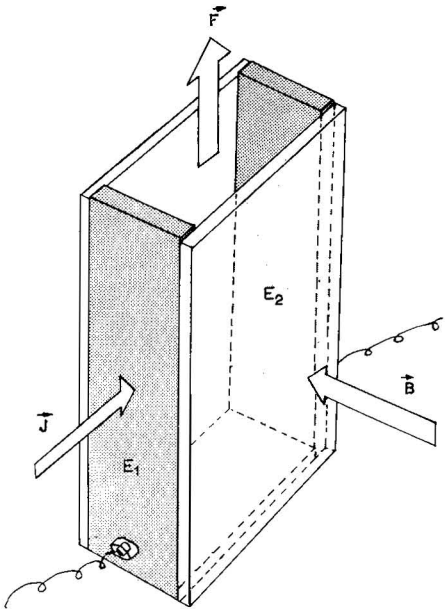

Fig. 6. Electromagnetophoresis cell. $\vec{J}$, Current density; $\vec{B}$, magnetic flux density; $\vec{F}$, electromagnetic force density; E_1 and E_2, electrodes. [From A. Kolin, *Biochim. Biophys. Acta*, 32 (1959) 538.]

and magnetic field are in phase, and sinusoidal of 60-Hz frequency. The above figures are r.m.s. values). The two gradients stabilize the liquid column against thermal convection which the high current density tends to engender. When we now reverse the current (*i.e.* its phase), we reverse the direction of the F vector, which now points upward as in Fig. 6. The configuration of the electromagnetic force density gradient is now unstable, while the stabilization by the density gradient is preserved.

To assess the combined action of the two gradients, we must add the gravitational and electromagnetic force densities at every point.

$$\vec{F} = (\vec{dg}) \pm [\vec{J} \times \vec{B}] \tag{4}$$

where $\vec{dg}$ is the gravitational force density (weight density) whose (downward) direction we designate as positive; $[\vec{J} \times \vec{B}]$ is the electromagnetic force density which can point up or down, depending on directions of $\vec{B}$ or $\vec{J}$. Since $\vec{J} = \sigma(\partial V/\partial x)$ (where $\partial V/\partial x$ is the potential gradient in the solution), we can write for eqn. 4

$$F = (dg) \pm B\,(\partial V/\partial x)\sigma \tag{5}$$

The stability thus depends on the gradient

$$\frac{\partial F}{\partial y} = g\left(\frac{\partial d}{\partial y}\right) \pm B\left(\frac{\partial V}{\partial x}\right)\left(\frac{\partial \sigma}{\partial y}\right) \tag{6}$$

(where $\partial V/\partial x = \text{const.}$).

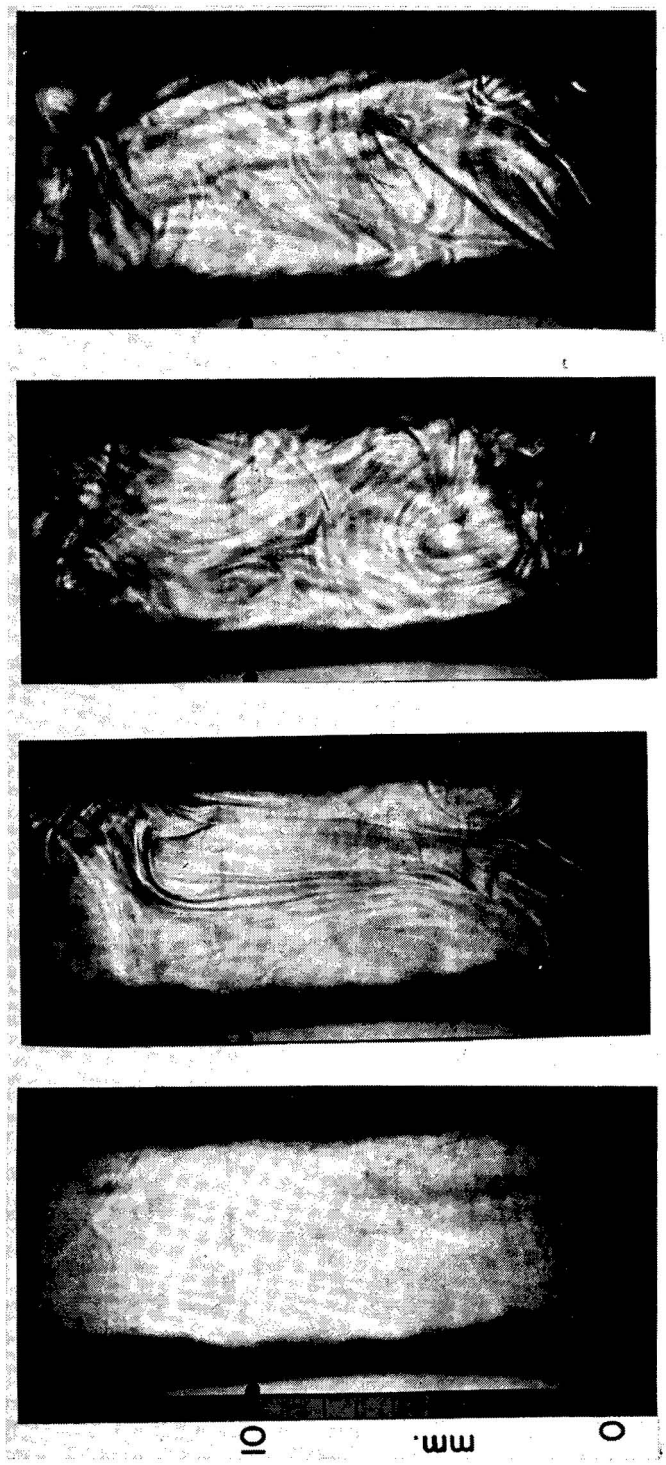

Fig. 7. Stabilization and destabilization of a density gradient by a superimposed conductivity gradient in a transverse magnetic field. (A) Stabilization of density gradient column reinforced by conductivity gradient in a suitably oriented (phased) magnetic field. (The electromagnetic force points in the directions of the density- and conductivity-gradients). (B), (C) and (D) The direction of the electromagnetic force is reversed by reversing the magnetic field or current. The electromagnetic force is now pointing in the direction of diminishing density and conductivity. The fluid column is destabilized as evidenced by turbulence revealed by "Schlieren" seen in photographs. [From A. Kolin, *Biochim. Biophys. Acta*, 32 (1959) 538.]

If in our concentration gradient where d and σ increase in the downward direction $\partial F/\partial y$ is positive (*i.e.* points downward), the resultant force density increases in the direction of the gravitational and electromagnetic forces in our example and the fluid column is stabilized. If the concentration distribution and the sign of the second term of equation (6) are such that $\partial F/\partial y = g(\partial d/\partial y) \pm B(\partial V/\partial x)(\partial\sigma/\partial y) = 0$, there is no stabilizing gradient. Finally, if the electromagnetic force $[\vec{J}\times\vec{B}]$ points in the direction of diminishing conductivity in our conductivity gradient (*i.e.* the second term of eqn. 6 is negative) and the absolute value of the negative second term of eqn. 6 surpasses the value of the first term, the column becomes unstable for $g\,|\,(\partial d/\partial y)\,| < |\,B(\partial V/\partial x)(\partial\sigma/\partial y)\,|$. Such a destabilization can be achieved by reversing the current or the magnetic field in an appropriately prepared combined g, σ gradient column.

It is worth adding that the surface of electrodes E_1 and E_2 of the cell in Fig. 6 could be coated with a very thin dielectric coating which would permit passing an alternating current of suitably high frequency between the electrodes without creating a short circuit precluding passage of a vertical direct current through the cell.

6. MAGNETOHYDRODYNAMIC CONVECTION—ENDLESS FLUID BELT ELECTROPHORESIS

6.1. Background of this development

Establishing density and/or conductivity gradients in the migration cell for stabilization against thermal convection introduced experimental and theoretical complications which I did not like. A stabilization method which I published in 1954[17], although mechanically more complicated, offered the possibility of performing electromagnetophoresis and electrophoresis in a fluid of uniform density. It involved rotation of a cell similar to that shown in Fig. 2 about a centrally located horizontal axis. This approach was based on the insight that thermal convection is a gravitational phenomenon and would not exist in a gravity-free space[17,25]. A particle entrained in a rotating cell will behave as if it were exposed to a rotating rather than unidirectional gravitational field. It will orbit with respect to the cell in a small circle about a stationary center instead of sedimenting to the cell bottom. A fluid element whose density is changed above the density of the ambient fluid by a temperature rise is analogous to a suspended particle and will be similarly inhibited from performing a unidirectional motion which would lead to thermal convection. It was thus possible to suppress thermal convection in such a cell rotating at a frequency of 1.3 r.p.s. while the NaCl solution was heated at the center of the cell by a current of 3 A passing between two electrodes 4 mm apart[17]. The rotation of the cell solved two additional problems in electromagnetophoresis: (1) it became unnecessary to adjust the density of the fluid to maintain particles in suspension and (2) the rotation of the cell within the magnetic field averaged out spatial variations in the field strength to which different portions of the cell were subjected, thus diminishing disturbances due to electromagnetic convection[18].

On my trip to Sweden in 1958 I saw an impressive independent development. It was Hjertén's rotating-tube electrophoresis apparatus in which rotational inhibition of thermal convection permitted obtaining beautiful electrophoretic zonal mobility spectra in the absence of stabilizing gradients[26]. Upon my return to Los Angeles, I

attempted to use the electromagnetic propulsion of my 1936 pumping experiment to replace the mechanical rotation of Hjertén's horizontal tube by electromagnetic rotation of the fluid in a stationary tube but failed to get satisfactory results. However, these experiments were not a total failure since they stimulated the following idea for stabilization against thermal convection in continuous-flow electrophoresis[11,25,27,28].

6.2. Principle of the method

Fig. 8a shows in cross-section an annular fluid volume sandwiched between two horizontal cylinders. The temperature of the inner cylinder is higher than that of the outer one. The resulting horizontal temperature gradient in the mid-plane of the annulus will tend to produce vortical fluid motion as indicated by a solid loop with arrows on the right side. The warmer fluid at the inner cylinder, being less dense will move upward while the denser fluid near the outer cylinder will move downward.

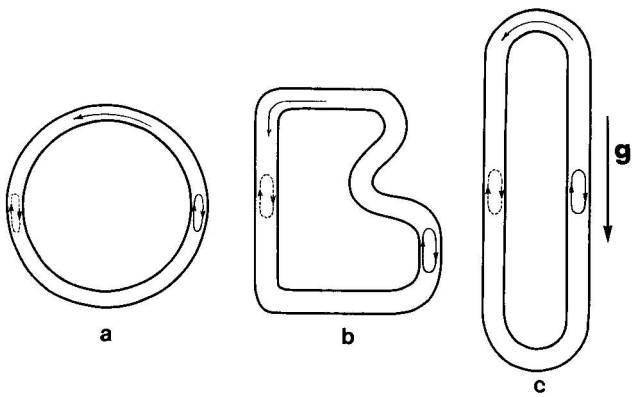

Fig. 8. Inversion of thermal convection vortices by fluid circulation in horizontal annular channels of different cross-sections (a, b and c). *g*, Gravitational field intensity. [From A. Kolin, in N. Catsimpoolas (Editor), *Methods of Cell Separation*, Plenum Publ. Co., 1977.]

Imagine now that we succeed in rapidly rotating the fluid in the annulus by 180° as indicated by the top arrow in the annulus. This will transfer our "solid" vortex into the position indicated by the dashed arrowed loop on the left annulus side. Due to inertia the fluid in the vortex near the inner cylinder will move downward and in the outer part of the vortex upward. These two directions of motion are, however, now opposite to the gravitational forces which generated the vortex and which still act on the fluid in it in the same directions; namely, upward on the warmer and specifically lighter fluid near the inner cylinder and downward on the cooler and specifically heavier fluid near the outer cylinder. Thus, the same forces which engendered the vortex on the right hand side of the annulus will retard and stop its rotation on the left hand side if we leave it there long enough. Since a certain amount of time is required to endow a vortex with its rotational kinetic energy, it is clear that we could effectively inhibit formation of thermal convection vortices by a slow uniform circulation of the fluid in the annulus as indicated by the top curved arrow.

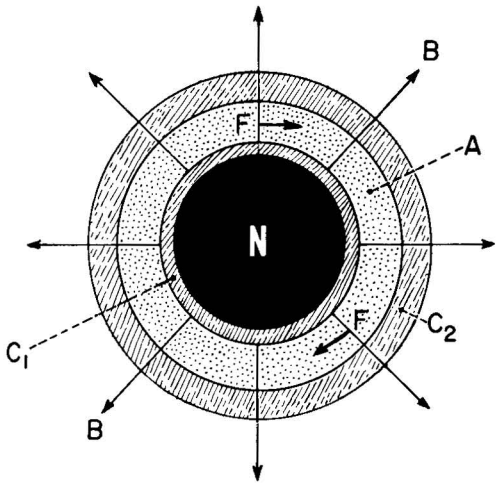

Fig. 9. Radial magnetic field (B) surrounding a cylindrical magnetic north pole (N) inside a buffer-filled annulus (A). C_1 and C_2, Walls confining the fluid in annulus A; F, electromagnetic force generated in the presence of a current flowing through fluid in A at right angles to the page. [From A. Kolin, in N. Catsimpoolas (Editor), *Methods of Cell Separation*, Plenum Publ. Co., 1978.]

It is obvious that the closed circulation path of the fluid need not be circular, but could have arbitrary shapes as those shown in Figs. 8b and 8c. It remains now to find a way to maintain such constant fluid circulation. Such a scheme is illustrated in Fig. 9. N represents in cross-section an isolated cylindrical magnetic north pole

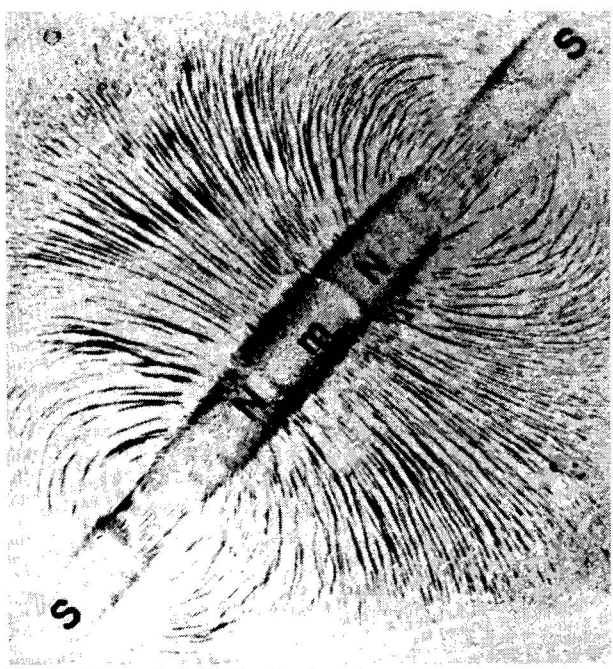

Fig. 10. Magnetic field distribution about a cylindrical soft-iron core m located between oppositely oriented cylindrical bar magnets (N, S). [From A. Kolin, *J. Chromatogr.*, 26 (1967) 164.]

and the vectors $\vec{B}$ its field lines. The pole is surrounded by two concentric plastic cylinders C_1 and C_2 between which is sandwiched an annular electrolyte volume. If we now imagine an electrical current passing through the electrolyte in the direction from the reader into the page, tangential electromagnetic forces (indicated by vectors $\vec{F}$) will be exerted upon the electrolyte, and the fluid in the annulus will be set into a very constant circular motion.

Since there are no isolated magnetic poles in nature, we must devise an approximation. Fig. 10 shows that a cylindrical soft-iron core m sandwiched between the north poles N of two cylindrical bar magnets NS exhibits a nearly radial magnetic field near its surface as is depicted in Fig. 9.

6.3. The circular endless belt apparatus

Fig. 11 translates this scheme into a simple instrumental arrangement. The magnets and iron core M are held inside the inner plastic cylinder C_1 which is surrounded by an outer plastic cylinder C_2 leaving an annular gap of 1.5 mm between them. The plastic cylinders are cemented to the electrode compartments EC_1 and

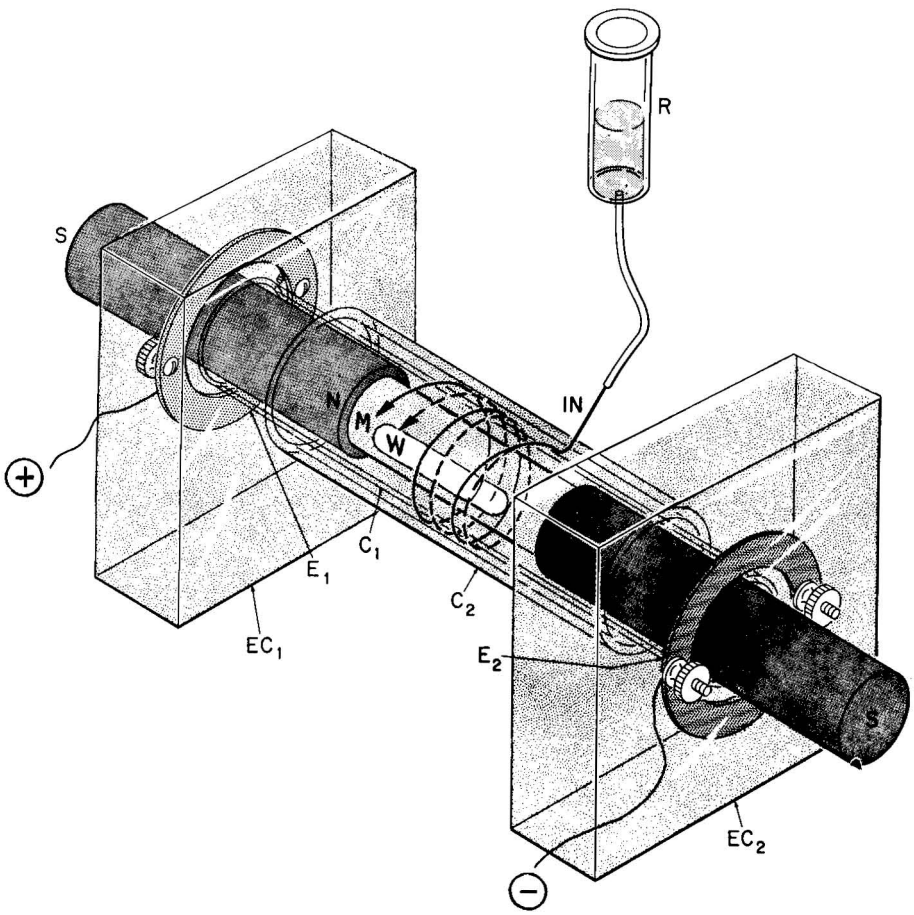

Fig. 11. Scheme of circular endless belt electrophoretic separator. N and S, magnet poles; M, soft-iron core; W, window in core M; C_1 and C_2, concentric plastic cylinders; EC_1 and EC_2, electrode compartments; E_1 and E_2, electrodes; IN, injector; R, sample reservoir. [From A. Kolin, *J. Chromatogr.*, 26 (1967) 164.]

EC_2 so that the latter communicate with each other through the annulus between C_1 and C_2. E_1 and E_2 are electrodes mounted in the compartments EC. If we now pour buffer solution into the cell and connect E_1 and E_2 to a current source, the interaction between the radial magnetic field and the axial current will cause the buffer in the annulus to rotate with clocklike regularity.

R is a reservoir containing the solution or suspension to be subjected to electrophoretic analysis. It delivers its contents through a fine glass capillary which passes through cylinder C_2 and terminates in the middle of the annular gap. If we inject, for instance, electrically neutral particles, they will be entrained in the circulating fluid and will accumulate in a circular orbit (in the absence of electro-osmosis). If on the other hand the particles are negatively charged, they will combine circular motion with axial electrophoretic migration toward the anode and their path will be a left-handed helix as shown in Fig. 11. The helical pitch is a measure of the electrophoretic mobility of the particles. Two ions or particles differing in electrophoretic mobility will follow divergent helical paths of different pitch as shown by the solid and the dashed helix in Fig. 11. Positive particles will migrate toward the cathode in a right-handed helical path. The window W is a gap in the soft-iron cylinder which permits passage of light through the core. In this fashion particles like cells and cell organelles can be visualized and photographed by dark-field illumination.

6.4. The non-circular endless belt apparatus

The use of a circular annular path is not favorable, especially for cell separations. Fig. 12A shows how cell sedimentation can move the injected particle stream toward the cylinders to which they could become attached. This can be remedied by using a non-circular "racetrack" for the fluid circulation. The scheme is the same as shown in Figs. 9–11, except that the cross-sections of the magnets, iron core and plastic enclosures are no longer circular. In Fig. 12B the particles move mostly in a vertical path in the central plane of the racetrack. When they move to the left under the core they sediment slightly below the midline and continue their upward journey

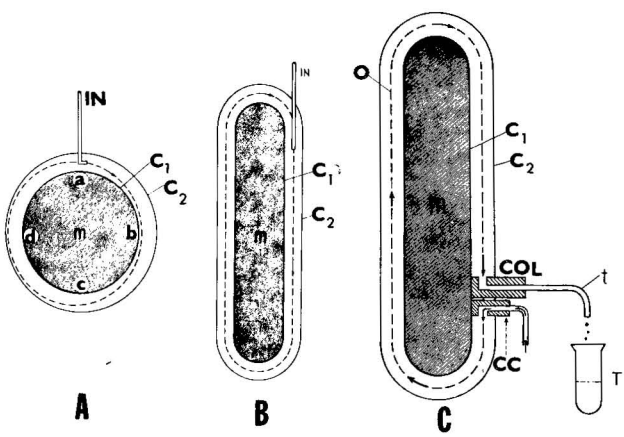

Fig. 12. Electromagnetic circulation of injected particles in annuli of different shapes. m, Iron core inside annulus; IN, injector; C_1 and C_2, walls confining fluid in annulus; COL, collector; t, collector tubing; CC, collector compensator; T, test tube. [From A. Kolin, in N. Catsimpoolas (Editor), *Methods of Cell Separation*, Plenum Publ. Co., 1978.]

left of the midline. However, as they move to the right above the core m, sedimentation brings them back toward the center of the racetrack so that they never get too far away from it. Fig. 12C illustrates how a collector COL can be installed at the end of the helical path to intercept the separated particles (or ions) and guide them to tubes T of a fraction collector.

Fig. 13 illustrates the separation space of the apparatus in a pérspective drawing. The iron core C (insulated with a layer of about 0.2 mm thickness of Epoxylite) harboring a quartz window W is centered (by small plastic spacers) with the mantle MA so that a gap of 1.5 mm between them surrounds the core C. The buffer solution which fills this non-circular annular gap is the endless fluid belt in which the continuous-flow electrophoretic separation takes place. Four permanent Alnico bar magnets of which only the N poles are shown generate the magnetic field which traverses the annular buffer belt substantially at right angles. The electrical current flows through the annulus in the direction of the arrows seen inside the tubes CP. The interaction of this current with the magnetic field maintains the buffer belt in a uniform circulation around the core C. The hollow core C is cooled by water entering and leaving through the cooling water pipes CP. The core thus cools the buffer belt from the inside. The outside of the buffer belt is also cooled. This is accomplished by milling out the front and back walls of the mantle MA leaving a thin plastic membrane to confine the buffer belt on the outside. Cooling water is circulated

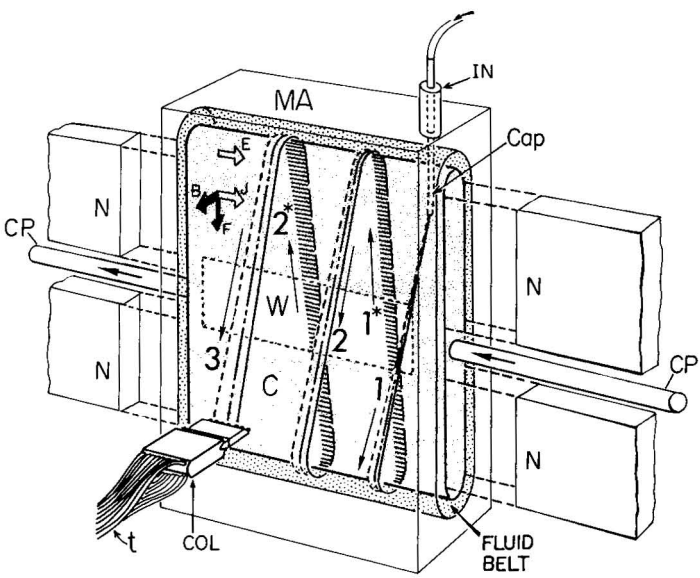

Fig. 13. Electrophoretic separation space of non-circular endless belt apparatus. N, north poles of magnets; C, hollow iron core; CP, cooling pipes carrying cooling fluid circulating through C; W, window in C; MA, mantle surrounding annulus (fluid belt); IN, injector; Cap, capillary; COL, collector; t, collector tubing; $\vec{E}$, electric field vector; $\vec{B}$, magnetic field vector; $\vec{J}$, current density vector; $\vec{F}$, electromagnetic force vector. 1* and 2*, first and second ascending streaks (behind the core C). 1, 2 and 3, first, second and third descending streaks (in front of the core C); L, light beam passing through the bottom of the annulus. [From A. Kolin, in N. Catsimpoolas (Editor), *Methods of Cell Separation*, Plenum Publ. Co., 1978.]

through the hollow cooling chambers thus produced in the mantle walls and cools the buffer belt by outward heat flow across the thin plastic membranes.

The sample of the material to be subjected to electrophoresis is injected at the center of the annular buffer belt thickness through a capillary of the injector (IN). The electrophoretically distinct components migrate in trajectories of different helical pitch and are intercepted at the end of their path by collector COL from which tubes t guide them to different test tubes.

6.5. Omission of membranes

While Fig. 13 shows the endless fluid belt and the magnets, it does not show the connection between the endless belt and the buffer and electrode compartments. This connection is illustrated in Fig. 14 which does not show the magnets and the core. The separation chamber shown in Fig. 13 is the section SCH of Fig. 14. In addition to the window W in the core there are two windows in the mantle, one in front and another one behind the core window. The electrodes E_1 and E_2 are in electrode chambers EC which are separated from the buffer chambers BC by plates P whose lower portions are perforated (as indicated by dashed lines). This is an

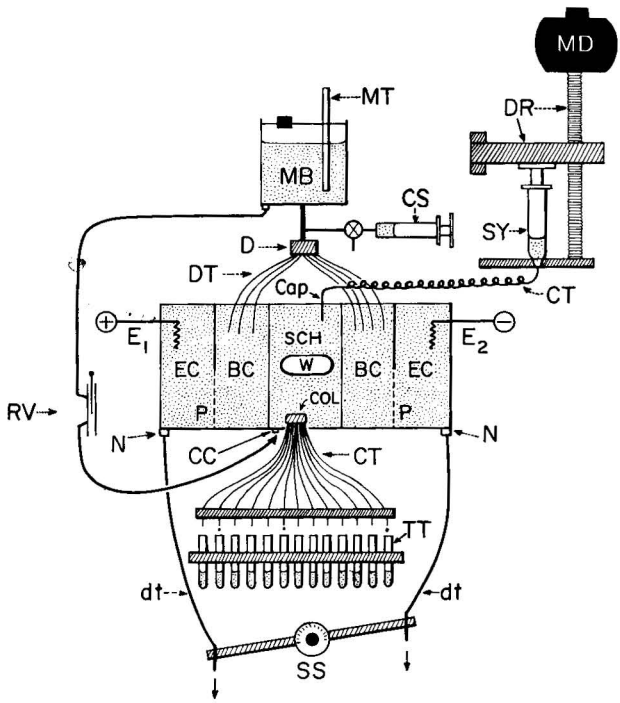

Fig. 14. Scheme of endless belt apparatus flow system. SCH, separation chamber; W, window; Cap, injector capillary; COL, collector; CT, collector tubing; CC, collector compensator nipple; TT, test tubes; BC, buffer compartments; EC, electrode compartments; E_1 and E_2, electrodes; N, nipples of rigid drainage tubes (not shown); dt, narrow plastic drainage tubes; SS, "see-saw"; P, perforated plates; MB, Mariotte bottle; MT, tube of Mariotte bottle; CS, clean-out syringe; D, distributor; DT, distributor tubes; CT, coiled tubing linking capillary to sample syringe; SY, sample syringe; MD, motor drive; DR, screw and plate which drive the plunger of syringe SY; RV, regulator valve for control of collector compensator inflow. [From A. Kolin, in N. Catsimpoolas (Editor), *Methods of Cell Separation*, Plenum Publ. Co., 1978.]

important distinguishing feature between the endless fluid belt apparatus and the free-flow electrophoresis apparatus[9,10], where the electrode chambers and the separation chamber must be partitioned by membranes to permit maintenance of a vertical pressure gradient in the separation chamber to drive the buffer through it. The electromagnetic propulsion does not require such a pressure gradient and thus permits omission of the membranes.

The significance of the omission of the membranes is as follows. The perforations in the plates P make it possible to prevent electrolysis products from migrating from the compartments EC into the separation chamber SCH. This is achieved by centrifugal buffer flow. Buffer is delivered into both buffer compartments BC at a constant pressure head from Mariotte bottle MB via distributor D through thin plastic tubes DT. This creates a rapid buffer flow through the small perforations in the plates P directed from the chambers BC toward the chambers EC so that when this flow is fast enough the electrolysis products cannot migrate toward the chamber SCH. This avoids spatial and temporal variations in pH and conductivity in the annular separation space which can be caused by membranes. The centrifugal buffer flow idea was first applied by Bergrahm to a column electrophoresis apparatus[29]. It was subsequently adapted to endless belt electrophoresis[12,30].

6.6. Axial buffer flow

In addition to the symmetrical centrifugal buffer flow, the scheme of Fig. 14 permits the imposition of an axial buffer flow through the annulus. This can be accomplished in step-wise fashion by transferring some of the tubes DT from the left to the right buffer chambers BC or vice versa. Usually one employs a surplus of tubes in the right BC chamber to maintain an axial buffer flow from right to left. A fine-control of axial buffer flow is accomplished by means of the "see-saw" SS which permits to lower the left outflow tube dt as the right one is raised, or *vice versa*. The collector compensator CC allows to return to the separation chamber (under the collector COL) an amount of buffer which enters the collector from the top. The clean-out syringe CS permits removal of air bubbles from the distributor system. The motor MD and drive DR expell the fluid to be analyzed from the syringe Sy via the tubing CT and capillary Cap into the endless buffer belt. A more detailed description of the instrument and its performance will be found elsewhere[31].

6.7. Illustrations of the performance of the circular endless belt apparatus

The following photographs illustrate the performance of the simple circular-path separator shown in Fig. 11. Typically the diameter of the annulus is about 3 cm, the annulus thickness 1.5 mm, the period of revolution of the buffer is about 22 sec at a current density of 10^{-2} A/cm^2 and magnetic field intensity of 150 gauss. Visible separations can be frequently seen within 5–10 sec[11].

Fig. 15 illustrates 15 helical turns of erythrocytes visualized by dark field illumination of the particle path through window W of Fig. 11[27].

Fig. 16 shows the separation of two microorganisms: *Saccharomyces cerevisiae* and *Rhodotorula*. Four consecutive helical turns are seen .The duration of migration from turn to turn is about 20 sec. We see how the separation between the particles gains equal increments with each turn[27].

Fig. 17 demonstrates an important stability property of the endless belt system.

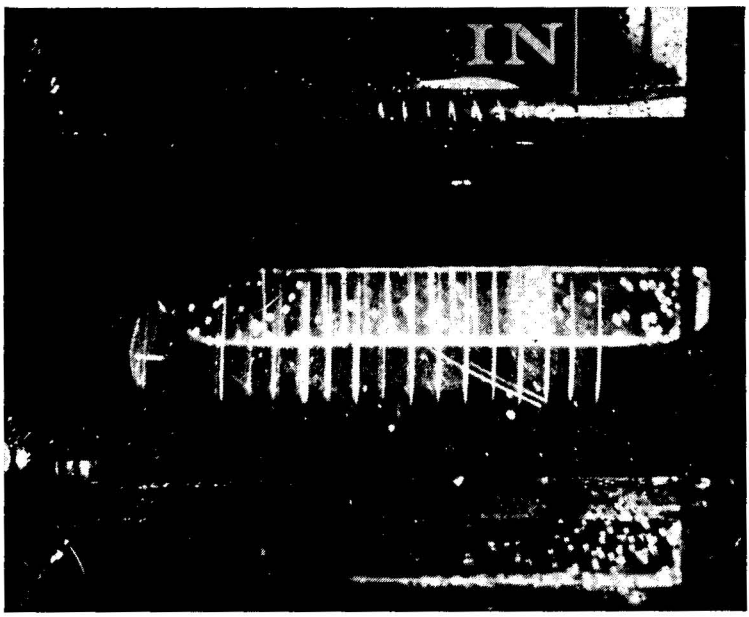

Fig. 15. Visualization by light scattering of a 15-turn streak of erythrocytes in circular endless belt. IN, Injector; W, window in core of central iron cylinder. The illumination is directed toward the reader (forward scattering). [From A. Kolin, *J. Chromatogr.*, 26 (1967) 164.]

The photos show several helical turns of the dye Evans blue: (a) at a voltage of 75 V between the cell electrodes and (b) after raising the voltage to 150 V. We see that the helical pitch has not changed although the electrophoretic migration speed has been doubled! The reason is the concomitant doubling of the rotational speed which allows only half the time for migration between the consecutive turns. In (c) the buffer has been diluted 1:2 to lower the electrical conductivity of the buffer while maintaining the same current (25 mA) as in experiment (a). The helical pitch is about doubled, because the voltage across the cell had to be increased while the constancy of current insured a constant rate of buffer rotation[11].

The separation between two components of a mixture increases with each helical turn, as we can see from Fig. 16. There is, however a limitation in this advantage since eventually the faster component after n turns my approach coincidence with the slower component of the $(n+1)$st turn. Such a fusion of previously well resolved components can be avoided by superimposing a lateral buffer flow in the direction of electromigration upon the electrophoretic migration. One can thus increase the helical pitch to any desired value and make wider separations possible. Fig. 18 illustrates changes in helical pitch by imposed lateral buffer flow. Initially, in (a), there is no lateral buffer flow. In (b) a lateral flow from right to left is initiated with consequent increase in pitch and in (c) the lateral flow is increased further[27].

Conversely, one can impose the lateral streaming in the direction opposite to electromigration. In this case one can obtain a non-isoelectric accumulation of a given component in a circular orbit. In Fig. 19 we see in (a) the initial helix of india ink. A lateral streaming arresting the right-to-left migration of india ink has been

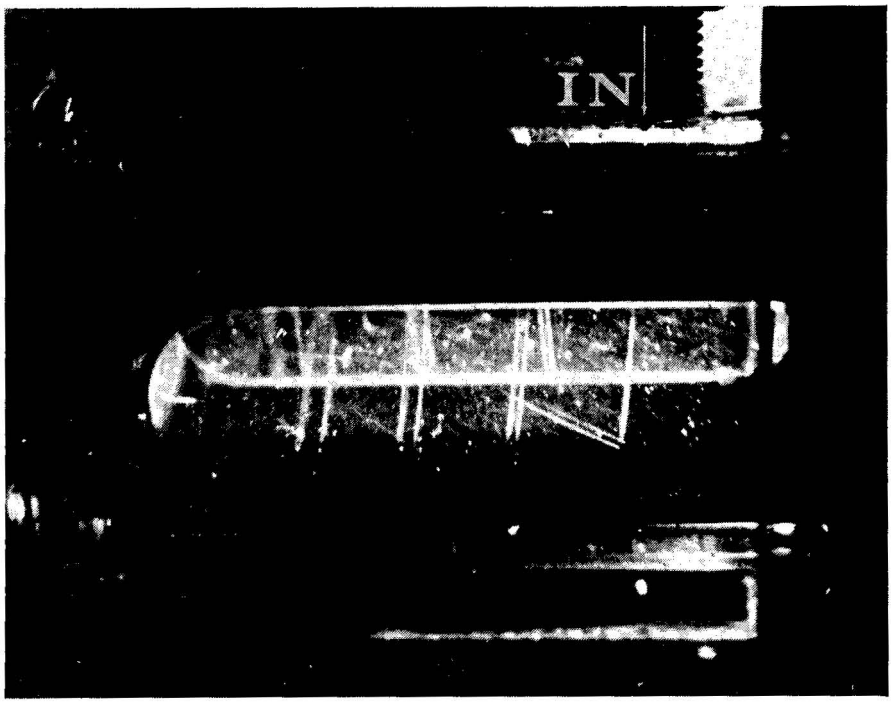

Fig. 16. Separation of two micro-organisms *Rhodotorula* and *Saccharomyces cerevisiae*, visualized by dark-field illumination. a, b, c, d, Consecutive helical turns; IN, injector. Arrow marks the point of sample injection. W, window in iron core. [From A. Kolin, *J. Chromatogr.*, 26 (1967) 164.]

imposed in Fig. (b) and the particles are accumulated in a circular orbit under the arrow[27].

Under certain conditions it may be advantageous to collect different electrophoretic components after a different number of turns. For instance, suppose we have 3 components of which the two faster ones are clearly resolved and get into different collector tubes, while the slowest component is so close to its neighbor that some of it enters the collector tube which intercepts its faster neighbor. Under these circumstances we can adjust the lateral flow so that the slowest component just misses the collector after n turns while its faster neighbors are intercepted by it in the nth turn. The slowest component then migrates one more turn and enters the collector in the $(n+1)$st turn ahead of the faster components and a considerable distance away from them in the collector[27].

Endless belt electrophoresis is not limited to continuous preparative separations. Discontinuous zone separations can be performed for micro-analysis. For this purpose we can inject a very fine streak (about 100 μm in diameter or less) of a mixture to be analysed. The streak may be as short as 1 cm or less and thus comprise a volume smaller than 0.1 μl. We can impose a lateral streaming to place the electrophoretically slowest zone into a stationary circular orbit allowing the faster components to migrate to the left until all components can easily be separated. The separate collection of the zones can be achieved by increasing lateral streaming so as to intercept the helical paths of the zones by the collector.

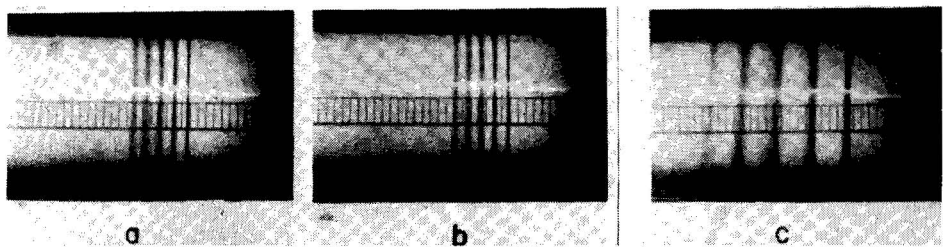

Fig. 17. Effect of voltage on helical pitch in circular endless belt apparatus. (a) Helix of Evans blue obtained at a current of 25 mA and a voltage of 75 V between the electrodes. (b) Helix of Evans blue after raising the voltage to 150 V. (c) Change in helical pitch at the current used in (a) after diluting the buffer 1:2. [From A. Kolin, *Proc. Nat. Acad. Sci. U.S.*, 46 (1960) 509.]

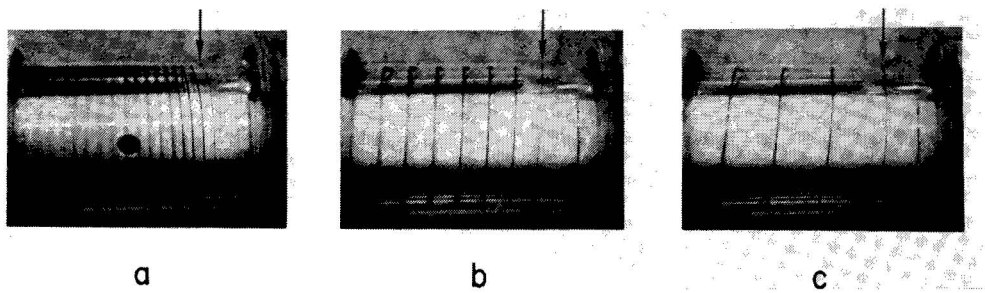

Fig. 18. Effect of lateral flow on helical pitch. The lateral flow is from right to left. The arrow marks the entry point of sample. The rate of lateral flow is increasing as we progress from (a) to (c). [From A. Kolin, *J. Chromatogr.*, 26 (1967) 164.]

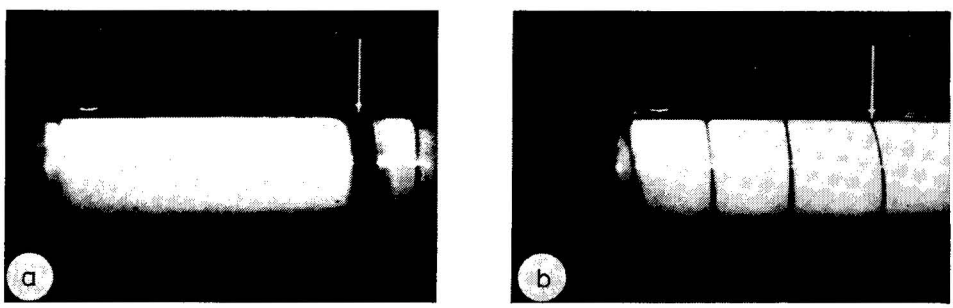

Fig. 19. Non-isoelectric accumulation in stationary orbit. (a) Helix of india ink injected at arrow. (b) Lateral flow is adjusted to accumulate india ink in a zero-pitch helix, *i.e.* circular orbit. [From A. Kolin, *J. Chromatogr.*, 26 (1967) 164.]

6.8. Illustrations of the performance of the non-circular endless belt apparatus

The dimensions of the annulus of the non-circular endless belt are about 10 cm in height, 9.5 cm in lateral length and 1.2 cm in width determined by the thickness of the iron core. The circumference of the racetrack is about 21 cm. The non-circular vertical endless belt is more effective than the circular one, not only with particles by inhibiting their sedimentation, but also with macromolecular solutions which can be injected at higher concentration without the hazard of the injected dense streak sedimenting excessively toward the walls of the core and mantle. The racetrack is long enough to yield adequate separations after the minimum

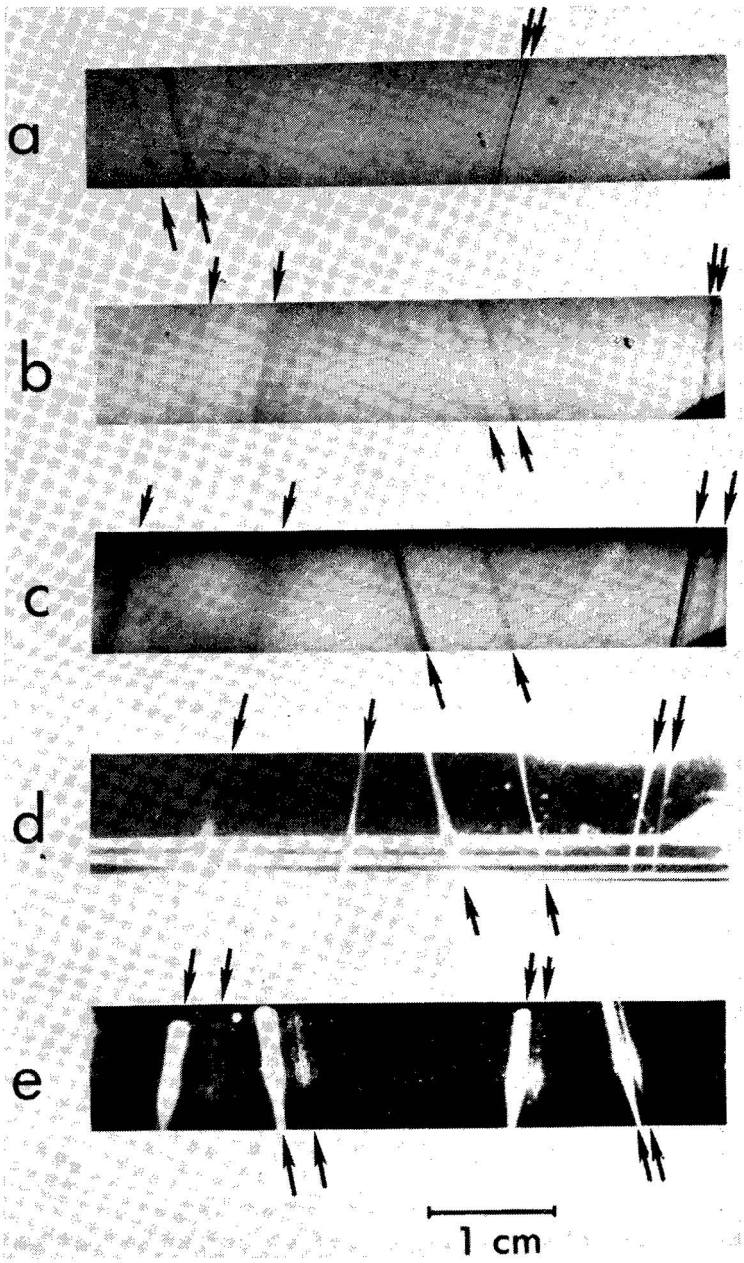

Fig. 20. Streaks in endless belt electrophoresis apparatus photographed (a) and (b) by ultraviolet light, (c) by visible light absorption, and (d) and (e) by light scattering. Current was 150 mA in (a) and 200 mA in all the others. (a) TMV strains U1 (left) and U2 (right), pH 7.1. (b) Human serum proteins, albumin (left) and γ-globulin (right), pH 8.75. (c) Mixture of bovine hemoglobin (left) and horse heart cytochrome c (right), pH 9.3. (d) *Rhodotorula* sp. (left) and *Escherichia coli* (right), pH 7. (e) Erythrocytes (left) and mainly granulocytes (right), pH 7.1. The streak pattern descending from the injector is out of the field of view. [From A. Kolin and S. J. Luner, *Anal. Biochem.*, 30 (1969) 111.]

number of $1\frac{1}{2}$ revolutions. It is seldom that more than $2\frac{1}{2}$ revolutions are required in practice.

The following figures illustrate the effectiveness of the vertical non-circular endless belt electrophoretic separator. Fig. 20 illustrates separations of blood cells, fungi, bacteria, viruses and macromolecules (proteins)[12]. In Fig. 20a we see the first descending and the first ascending portions of the non-circular helical paths of two strains of tobacco mosaic virus (electrophoresis proceeds from right to left). The higher-mobility strain U1 is less abundant than the slower migrating strain U2 in the mixture. The separation seen in the first double streak was achieved about 8 sec after injection into the annulus and the 3-mm separation seen in the second double streak on the left resulted after *ca.* 0.5 min electromigration. The mobility difference between these virus strains was 15 TU. They were visualized by ultraviolet photography which was also used to photograph the separation between human serum albumin (left streak) and γ-globulin (right streak) shown in Fig. 20b.

Visible-light photography was used to record the separation of a mixture of bovine hemoglobin (left streak) and horse heart cytochrome *c* (right streak) into two streaks shown in Fig. 20c.

The splitting of a mixture of the yeast *Rhodotorula* (left) and *Escherichia coli* (right) seen in Fig. 20d was visualized by dark-field illumination as was the separation of erythrocytes (faster component) and granulocytes (slower component) illustrated in Fig. 20e.

Fig. 21 shows a rapidly achieved (about 50 sec) wide separation pattern of a mixture of 3 bacteria: *E. coli*-ML35 strain (a), *Proteus vulgaris* (b) and *E. coli*-BE30 strain (c). It is interesting to note that *Proteus vulgaris* splits into two electrophoretically distinct components b_1 and b_2.

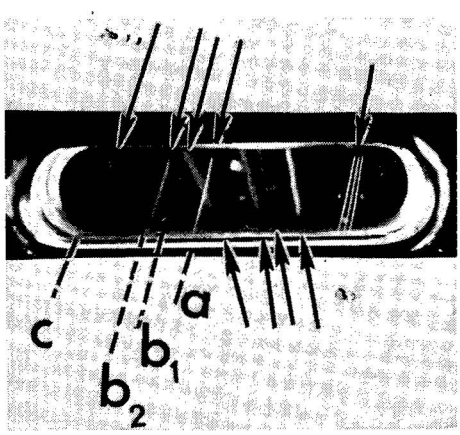

Fig. 21. Separation of 3 bacteria with endless belt. (a) *E. coli*-ML35 strain. (b_1) and (b_2) Two components of *Proteus vulgaris*. (c) *E. coli*-BE30 strain. [From A. Kolin, in N. Catsimpoolas (Editor), *Methods of Cell Separation*, Plenum Publ. Co., 1978.]

Finally, in Fig. 22 we see the separation of mouse mesenteric lymph node cells into two electrophoretically distinct components.

The main usefulness of endless belt electrophoresis lies in the field of particle separations which could not be carried out very effectively and conveniently by

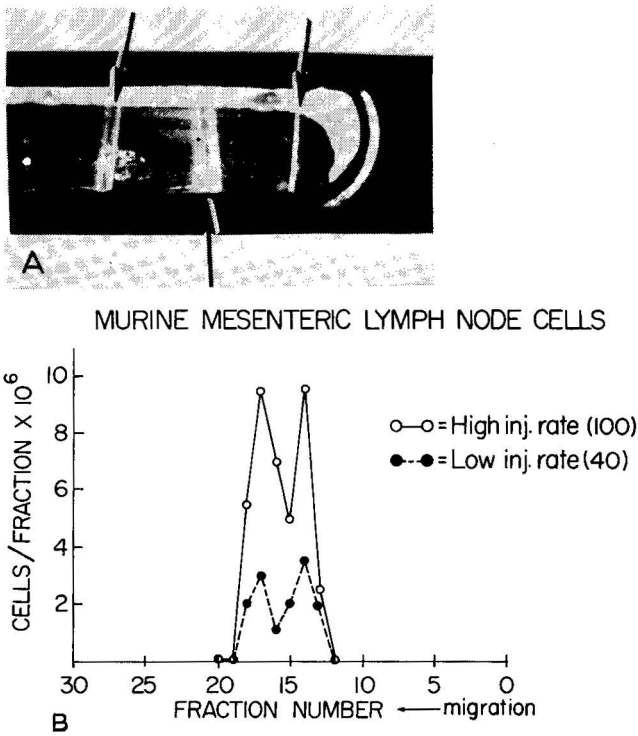

Fig. 22. Separation of murine mesenteric lymph node cells into two components. A, Photograph of separation pattern; B, collection patterns at high and low sample injection rates. [From A. Kolin, in N. Catsimpoolas (Editor), *Methods of Cell Separation*, Plenum Publ. Co., 1978.]

electrophoresis in supporting media. Although endless belt electrophoresis of macroions is very convenient, it cannot compete with the resolving power of gel electrophoresis.

6.9. *Resolving power*

The theory of resolution in endless belt electrophoresis and free-flow fluid curtain deviation electrophoresis is essentially the same. It has been worked out for endless belt electrophoresis[28] and utilized by workers using fluid curtains[10,32]. We shall proceed therefore for simplicity as if we were dealing with a flat fluid sheet and make reference to the shape of the endless belt only wherever it is necessary.

In the preceding discussion we were tacitly assuming a uniform flow of buffer in the fluid belt and absence of electro-osmosis. Under such unrealistic ideal conditions and in the absence of diffusion the resolution criterion is self-evident. An injected streak of circular cross-section containing two separable components will simply split into two streaks which will be considered as resolved as soon as they no longer overlap. Actually, the curtain flow velocity profile is parabolic and an injected circular streak is distorted in cross-section as shown in the photograph of Fig. 23[30,33]. The photograph has been taken looking end-on at the helical streak of an endless belt apparatus as it turns over the top of the core C. The originally circular streak cross-section is distorted into a crescent-like shape. The electromigration is from

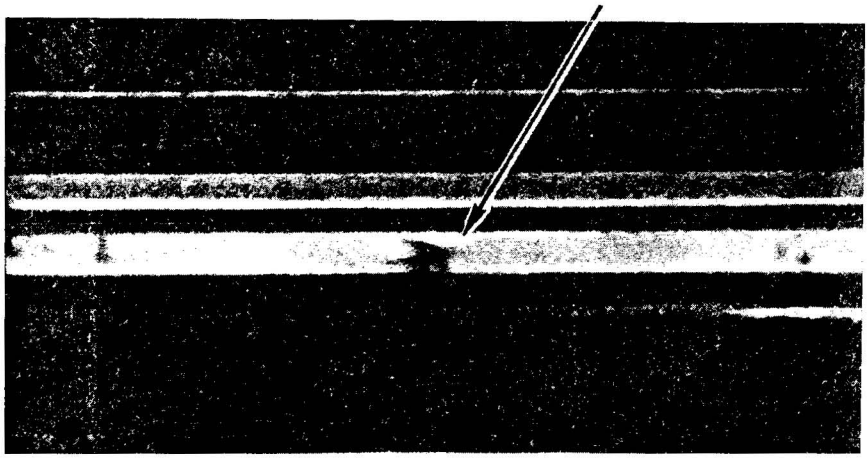

Fig. 23. "Crescent" distortion of an originally circular streak cross-section. End-on view of streak at the top of endless belt annulus. [From S. J. Luner, *Thesis*, Univ. of California, Los Angeles, Calif., 1969.]

right to left. Thus, the "horns" of the crescent contain particles which have advanced further to the left than those at the annulus center. As we shall see below, this does not mean that the electromigration velocity is smaller at the center than near the walls of the annulus.

We can visualize the process of streak distortion as follows. The white stripe in Fig. 23 at the center of which the crescent is located is the cross-section of the fluid curtain which we imagine flowing in the direction from the reader toward the page. Assume that a circular streak of negative particles starts its journey through the curtain at the eye level of the reader. They migrate to the left toward the anode as they move toward the page in the laminar flow within the fluid curtain. In view of the parabolic velocity profile in the laminar flow, the fluid velocity toward the page will be greatest at the center of the fluid curtain and will diminish toward zero at the walls. Therefore the centrally located particles will require less time than non-central particles to reach the plane of the page and will have been, consequently, migrating to the left a shorter time, than the particles near the walls confining the curtain. These particles will require a longer time to reach the plane of the page and, hence, will spend more time in electromigration to the left and will be deviated more in that direction. In view of the fact that this distortion results from the combination of the uniform electrophoretic velocity distribution of the particles with a parabolic flow velocity distrubution and because of the resulting divergence of the trajectories of the centrally located "rearguard" of the particle streak and of the outermost particles nearest the curtain walls, I called this phenomenon "parabolic divergence"[28,31]. This phenomenon has nothing to do with electro-osmotic streaming, in fact, the latter can be used to remove parabolic divergence so as to approximate ideal freedom from streak distortion, as we shall see below.

The distribution of the velocity u in the fluid curtain is

$$u = u_0 \left[1 - 4\left(\frac{z}{h}\right)^2\right] \tag{7}$$

where u_0 is the central maximum velocity, z is the distance of a point from the mid-plane of the curtain and h the thickness of the fluid curtain[11,28,31]. If we combine this vertical curtain flow with a transverse parabolic lateral flow v' (as it is used in the endless belt apparatus)

$$v' = v_0' \left[1 - 4\left(\frac{z}{h}\right)^2\right] \tag{8}$$

we obtain a resultant motion in which the fluid velocity will deviate from the vertical by the same angle of inclination i regardless of the value of z:

$$\tan i = \frac{v'}{u} = \frac{v_0'}{u_0} \cdot \frac{1 - 4\left(\frac{z}{h}\right)^2}{1 - 4\left(\frac{z}{h}\right)^2} = \frac{v_0'}{u_0} = \text{const.} \tag{9}$$

Since the coordinate z cancels out, an injected streak as thick as the fluid curtain will be deviated at all points of its cross-section by the same angle i and there will be no crescent distortion[28,31].

On the other hand, streak distortion will occur if the vertical flow represented by eqn. 7 is combined with a lateral motion of uniform velocity over the thickness of the curtain[28,31]. Since electro-osmosis is usually present under the experimental conditions of electrophoresis we shall include this fluid motion in our equation for horizontal particle velocity (in the absence of imposed lateral laminar flow). The electro-osmotic velocity (v) distribution is given by[11,31]

$$v = \frac{f_y}{2\eta}\left[z^2 - \left(\frac{h}{2}\right)^2\right] + v_w \tag{10}$$

In this equation f_y is the force density responsible for electro-osmotic streaming, η the fluid viscosity, and v_w the fluid velocity at the walls due to electro-osmosis. If we now add to this horizontal velocity the particle velocity due to electrophoresis $v_e = -\mu E$ (where μ is the electrophoretic mobility and E the electric field intensity), we obtain the resultant horizontal particle velocity

$$v^* = \frac{f_y}{2\eta}\left[z^2 - \left(\frac{h}{2}\right)^2\right] + E(W - \mu). \tag{11}$$

The term W is the electro-osmotic mobility, *i.e.* fluid velocity at the curtain wall at unit electrical field intensity and μ is the electrophoretic mobility of the particles.

The angle i of streak deviation will now be given by

$$\tan i = \frac{v^*}{u} = \left(\frac{f_y}{f_x}\right) \frac{z^2 - \left(\frac{h}{2}\right)^2}{z^2 - \left(\frac{h}{2}\right)^2} + \frac{E(W - \mu)}{z^2 - \left(\frac{h}{2}\right)^2} \tag{12}$$

or

$$\tan i = f_y/f_x + \frac{E(W-\mu)}{z^2 - \left(\frac{h}{2}\right)^2} \tag{13}$$

where f_x is the vertical force density responsible for maintaining the vertical curtain flow[11,31]. The coordinate z occurs now only in the right-hand term. We see that this term vanishes for $W = \mu$. The angle of streak deflection becomes then independent of z (ref. 11):

$$\tan i = f_y/f_x = \text{const.} \tag{14}$$

It was thus recognized in 1960 that the parabolic divergence (streak distortion) vanishes for one particular particle species, namely that particular electrophoretic component whose electrophoretic velocity equals the electro-osmotic velocity at the wall[11]. The streak corresponding to this particle species in an electrophoretic separation spectrum will be free of parabolic divergence, even if the streak diameter is as large as the thickness of the curtain. All the other components of the mixture will have streaks which will deteriorate by broadening[11].

This compensation phenomenon would not be very useful without the ability to change at will the electro-osmotic mobility W of eqn. 13. With this capability, one could adjust W to remove streak distortion from any electrophoretic component and optimally collect it in a wide streak. Such a possibility has been described in an ingenious suggestion[32] to modify in controlled fashion the average value of the wall zeta-potential. However, even if fully successful, this idea would permit in analysis of a mixture only one component at a time to be collected under favorable conditions. An entirely different approach offers the possibility of simultaneous streak collimation for all electrophoretic components and thus achievement of optimal resolution at maximal throughput[31,34,35].

6.10. Preparative resolution

We define the preparative resolving power R in streak deflection electrophoresis as the ratio between the mobility difference $\Delta\mu$ of two particle species which can be distinguished (and collected) as two separate components and the mean of their electrophoretic mobilities, $\bar{\mu}$ (ref. 28):

$$R = \frac{\bar{\mu}}{\Delta\mu} \tag{15}$$

It has been shown[11] that the preparative resolution of the endless belt of thickness h as well as the free-flow curtain apparatus is given by

$$R = \bar{\mu}/\Delta\mu = (h/d)^2 \tag{16}$$

We can thus achieve any desired degree of resolution by making the streak diameter d suitably small[11]. For instance, by using an injector yielding a streak diameter $d = 0.15$ mm in a curtain or annulus of $h = 1.5$ mm thickness, we obtain from

eqn. 16 $\Delta\mu \approx 10^{-2}\,\bar{\mu}$ and we can resolve two ionic species differing by 1% in their electrophoretic mobilities.

In practice, where high throughputs are often essential one must make a compromise between the conflicting requirements of high throughput (thick streak) and high resolution.

6.11. Mobility measurements

The above considerations of resolving power apply only to preparative separations. In analytical applications like measurement of the electrophoretic mobility, streak distortion is not a problem. The central edge of the streak (the trailing edge in Fig. 23) is very sharp. If we confine our attention to this edge, we are dealing in effect with an infinitely thin well centered streak.

For mobility measurements in the endless belt one needs a baseline of zero mobility as a reference. This can be obtained by injecting an uncharged dye like Apollon whose streak provides such a baseline which is valid in the presence of electro-osmotic and lateral laminar streaming. For absolute mobility measurements, we inject a bolus of dye and measure the period of revolution. Then the deviation from the baseline streak of the streak of the particles whose mobility is being measured is determined after one revolution. This gives the absolute value of electrophoretic velocity. The electrical field intensity is obtained by measuring the potential difference between the ends of the annulus of known length and the mobility follows then as the ratio of the velocity and field intensity.

Relative mobility measurements are simpler. The mobility of a dye (brilliant blue) which does not change significantly over a wide pH range is determined once and for all. This dye is then injected along with the material under study[12]. The ratio of the distances of the two streaks from the zero-mobility reference streak at any point of the helical path is equal to the mobility ratio of the two materials under comparison. Since one of them is a substance of known mobility, a photograph of the separation pattern provides a measure of the unknown mobility.

7. CONCLUSION

Non-uniform magnetic fields exert forces upon particles submerged in a fluid of a magnetic susceptibility different from theirs. Establishing a gradient in magnetic susceptibility within the fluid in a non-homogeneous magnetic field offers the possibility of focusing particles in zones where the magnetic susceptibility of the fluid is equal to that of the particles (isomagnetic focusing). Thus, there exists the possibility of sorting particles in an "isomagnetic spectrum" in accordance with differences in their magnetic susceptibility values.

Inversion of the electromagnetic flowmeter effect results in electromagnetic pumping action. Such magnetohydrodynamic propulsion can be applied to an endless fluid loop maintaining it in constant circulation. This mechanism of fluid propulsion has the advantage that electromagnetic forces act upon each fluid element and that no pressure gradients have to be used to propell the fluid. As a result, a continuous-flow separation system can be achieved which does not require lateral membranes to create a closed channel for the flow of the buffer curtain. The omission of membranes eliminates drifts and spatial and temporal changes in pH and electrical conductivity

within the separation space of the fluid ribbon. In addition, the revolution of the buffer about a horizontal axis suppresses thermal convection and the particles to be separated which are orbiting in the circulating endless belt gain repeatedly constant increments in separation with each revolution. Thus, the particle path is wound up like a wire in a coil around an iron core and a long migration path can be accomodated in a compact apparatus.

Instead of generating fluid circulation, the combination of a current in an electrolyte with a perpendicular magnetic field can be used to generate a hydrostatic pressure gradient. Coupling of this hydrostatic force with a suitable gradient in electrical conductivity in the electrolyte column can be used to achieve stabilization against thermal convection similarly to density gradient stabilization. By superimposing such an electromagnetically activated conductivity gradient upon a density gradient, one can greatly augment the latter's effectiveness in stabilizing the fluid against thermal convection.

The combination of an electric current in a fluid with a perpendicular magnetic field makes it possible to generate in a closed body of fluid effects analogous to gravity and buoyancy. Particles differing from their surroundings in electric conductivity experience forces at right angles to the magnetic field and current. These forces vanish in an equiconductive environment and reverse their direction as the particle conductivity changes from a lower to a higher-than-environmental conductivity value. For biological cells the conductivity parameter is highly variable from one cell species to another, which makes this effect (electromagnetophoresis) of special interest for biological cell separations. In a gradient of electrical conductivity, electromagnetophoresis can sweep particles from all parts of a column toward zones where the electrical conductivity is equal to that of the particle. Such iso-conductivity focusing is analogous to isoelectric focusing and more closely so to isopyknic focusing in density gradients. Among the aspects of electromagnetophoresis which may make it particularly attractive for biological cell separations are the possibilities (a) of using alternating currents (with alternating magnetic fields), (b) of conducting cell separations in biologically compatible media like blood serum, (c) of separating cells according to size and shape differences and finally (d) the feasibility of cell separations according to differences in two highly variable parameters: electrical conductivity and ionic cell membrane permeability. Electromagnetophoresis remained practically unused since 1954. There is a good likelihood that availability of superconducting magnets will stimulate its application to separations of cells and subcellular particles.

8. ACKNOWLEDGEMENTS

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9. SUMMARY

The idea of the electromagnetic flow meter suggested experimental configurations which led to the ideas of electromagnetic pumping, electromagnetophoresis, electromagnetic stabilization against thermal convection, endless fluid belt electro-

phoresis, isoconductivity focussing, isoelectric focussing, isodielectric focussing, isomagnetic focussing, and, in general, isoperichoric focussing effects. Electromagnetophoresis is an effect which can be obtained in alternating or constant crossed electric and magnetic fields. Particles whose electrical conductivity differs from that of the surrounding fluid are set in motion irrespective of their electric charge (which may be zero). Separation effects are based on differences in the particles' electrical conductivities, size, shape and electrical membrane properties. In endless fluid belt electrophoresis electromagnetic pumping action maintains fluid circulation in an annular conduit in which axially electromigrating particles describe a helical path at the end of which they are intercepted and collected. Ideas of isoconductivity focussing, isoelectric focussing, isodielectric focussing, and isomagnetic focussing are byproducts of the aforementioned ideas.

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CONTINUOUS FREE-FLOW ELECTROPHORESIS AS AN ANALYTICAL AND PREPARATIVE METHOD IN BIOLOGY*

KURT HANNIG
Max-Planck-Institut für Biochemie, 8033 Martinsried (G.F.R.)

CONTENTS

1. INTRODUCTION

Within the last few years, continuous free-flow electrophoresis has proved to be a mild, rapid and efficient method for preparative separation problems. It not only permits the separation of soluble substances such as proteins, but also the separation and isolation of viable cells, or cell organelles and membrane systems.

As the principle of electrophoresis uses differences in the surface charge of particles for their separation, its special significance lies in the investigation of those biological processes which are related to a change in the cell surface structure. The outer cell membrane is the communication organ of the cell. Changes which occur at the micro-levels of their molecular structure are mostly the expression of differentiation and transformation events or are diseased abnormalities that can lead to the blocking of information exchange. Most of the components of the cell surface, and of biological membranes in general, represent carriers for different electrically charged groups. They are often the loci of specific functions. Separation techniques such as electrophoresis, which use these different functional properties for the separation of biological particles from mixtures, can be useful tools in membrane and cell research. In this paper, some of the latest results obtained with free-flow electrophoresis are presented.

* Dedicated to the 75th birthday of Professor A. Butenandt.

2. EXPERIMENTAL

2.1. Apparatus for preparative separation

In a narrow separation chamber (55 × 10 cm) a buffer film of thickness 0.5–0.6 mm flows in a laminar manner from top to bottom[1,2]. An electric field is set up perpendicular to the buffer stream.

The samples to be separated are injected continuously into the streaming medium through one of the openings in the upper part of the chamber. Particles with different electrophoretic mobilities move along different paths and can be collected continuously at the bottom end of the chamber by a 90-channel peristaltic pump. The tubes lead to a fraction-collecting container. A very effective cooling system permits the application of high field strengths of 100–120 V/cm, and also a relatively high buffer streaming velocity. The biological material remains in the electric field for only 3–7 min, according to the result required.

With this system, all requirements that are necessary for a careful isolation of biological cell material, including sterile conditions, can be fulfilled, and 200–600 million cells or 20–50 mg of membrane or organelle proteins can be separated per hour. There is no loss of cell vitality as a result of the separation process.

2.2. Apparatus for analytical separation

As a continuously working method, free-flow electrophoresis offers both high throughputs for preparative purposes and a rapid change of difficult samples while maintaining constant conditions for analytical separations. In the latter instance, high sample throughput is unnecessary, but it is essential to increase the resolution by shortening the separation periods. Hence the basis is given for automation of analytical test series, and an analytical separation device based on the principle of free-flow electrophoresis, with the use of optical densitometry by means of slit scanning, has therefore been designed[3]. The detection system permits absorption measurements to be made in the ultraviolet range. The usual analytical separations of serum protein for clinical diagnostics, cell separations and mobility measurements can be carried out in less than 1 min per sample.

The separation chamber gap for analytical purposes has a width of only 3 cm, a height of 18 cm and a depth of 0.3 mm. Because of the high thermal stability and simple procedure for filling the separation chamber, the buffer flows from bottom to top. For the optical detection of the separated sample components through the window of the separation chamber a slit scanning photometer is used. Because of the small transmission differences, a high luminance, a low-noise photomultiplier with high UV sensitivity and special measurement electronics are required. A schematic diagram of the device is shown in Fig. 1.

A 200-W deuterium lamp serves as a light source. Via a monochromator, the light beam is moved continuously back and forth across the separation chamber window by means of mirrors. The output signal from the photomultiplier is amplified and noise is filtered by low passes. For a better determination of the small transmission differences, the bias light intensity is subtracted during each scan. This difference signal is amplified and displayed on-line on a storage monitor, or fed into a data-processing

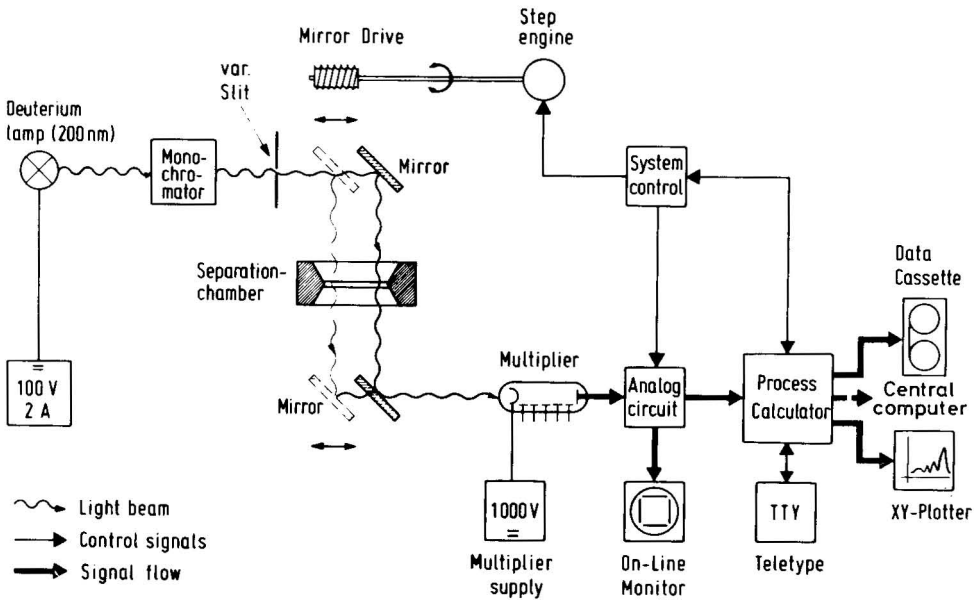

Fig. 1. Schematic diagram of analytical free-flow electrophoresis.

system. A process control computer averages several scans and carries out a background subtraction. The processed distribution curves can be plotted and stored on data cassettes for further processing in central computers. All functions work automatically.

3. RESULTS AND DISCUSSION

3.1. Preparative separation

3.1.1. Separation of cells

In an immune system, it is of great interest to investigate the correlations between the differentiating cells and their environment. The starting point for such investigations was our finding that free-flow electrophoresis permits the separation of immunocompetent peripheral lymphocytes into a population of thymus-dependent T-cells and into another population of thymus-independent B-cells[4–9]. These results, first obtained with rodents, were completed by experiments on baboons and chimpanzees[10–12]. This implies the possibility of studying immune mechanisms on human T- and B-cells.

By combining electrophoretic separation with that of 1-*g* sedimentation, it was possible to isolate five T-cell populations from thymus with high concentrations and to define them in terms of their biological significance[13,14]. These cells are constitutents of a differentiating sequence, which is characterized by increasing maturity of its members. The cell populations thus isolated show functional differences, which permit a useful classification and characterization of its functions[15].

The successful separation and isolation of large numbers of functionally viable

B- and T-cells and their subpopulation or precursor cells[16–19] is of practical medical significance. The understanding of the stage of differentiation during which and the conditions under which immunocompetence or tolerance appear would make possible the systematic manipulation of the immunity tolerance limits, which would be of great significance not only for the treatment of disease but also for transplant surgery.

In addition to the separation of immunocompetent cells, new possibilities arise in the preparation of a homogeneous cell population from a cell suspension from kidney cortex by using free-flow electrophoresis[20,21]. The kidney cells produce a number of physiologically important substances that control metabolic processes. For example, the enzyme renin has been shown to play an important role in the Na^+ and water metabolism of the organism, as it can activate the angiotensin-I–angiotensin-II–aldosterone system. As a consequence, it is involved in certain types of hypertension.

Many questions have been answered concerning the physiology of renin. However, its biosynthesis (its pathway within the cell) and its proliferation from the cell are still unclear. Therefore, it would be an important step to be able to isolate viable renin-active cells. From a cell suspension of kidney cortex cell (rabbit), several homogeneous populations could be isolated[20,21]. Proximal and distal tubule cells have been characterized by their morphology, and another population has been demonstrated to be renin-active (radioimmunoassay). The biochemical characterization of these cells is being investigated, and also the vitality of the cells, which in culture incorporate uridine linearly over a period of 8–12 h. This vitality makes it possible to carry out stimulation and blocking experiments in the isolated cell populations.

3.1.2. Organelles and membranes[22]

Not only the isolation of viable cells, but also the purification of their membrane systems and cell organelles such as lysosomes[23,24] is of importance. Electrophoresis gives experimental evidence of the high purity isolation of these particles and an accurate characterization according to the electrical surface charge.

Great importance is also attached to the achievement of a better knowledge of the other ("interior") side of a membrane. New possibilities arose after it became feasible to turn the membrane surface partly inside out and thus obtain particles the hidden "inside" of which then is located on the outside. The preparative separation of such "inside out" vesicles in a centrifuge according to differences in density is very difficult, if not impossible. However, in most instances the membrane surfaces of the "inside" and "outside" have different electrical charges, the difference being great enough to permit a separation by electrophoresis and isolation in a pure condition.

Such investigations have been carried out successfully with mitochondrial membranes[25,26], erythrocytes[27] and plasma membranes[22]. As an example, experiments with erythrocyte membranes are considered below (Fig. 2).

On the basis of several previous investigations, it is assumed that the erythrocyte membrane can form vesicles with the original inner surface on the outside. As intact erythrocytes carry almost all of their sialic acid on the outside of their cell membranes, the two sides of the membrane should have different electrical surface properties, making electrophoretic separation from each other possible.

After electrophoresis, two fractions of membraneous material were obtained from erythrocyte ghost preparations. The left peak represents vesicles that have the same electrophoretic behaviour as intact erythrocytes and that also have their sialic

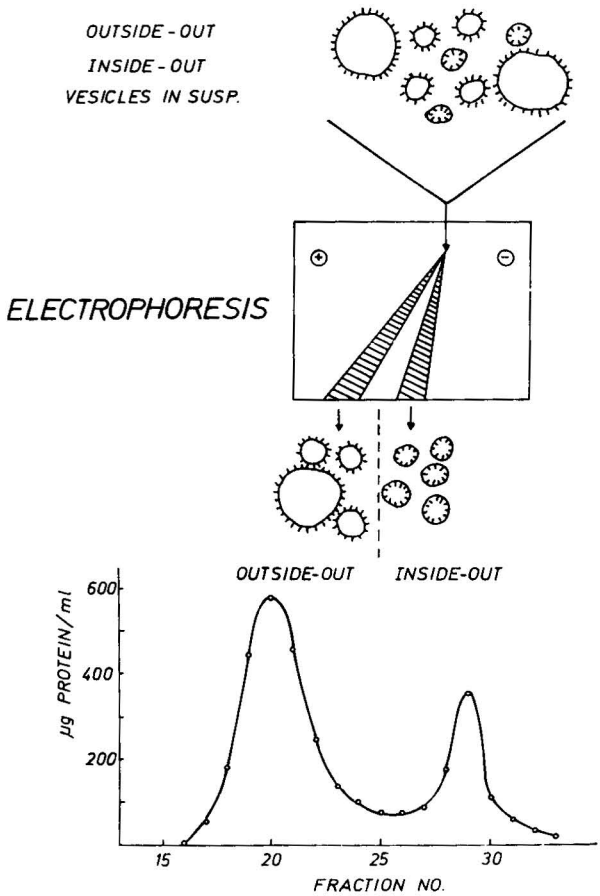

Fig. 2. "Inside-out vesicles"[27]. The upper part of the figure shows a schematic diagram of a separation of "inside-out" from "outside-out" vesicles. The lower part illustrates the original distribution curve of "outside-out" (left peak) and "inside-out" (right peak) vesicles of erythrocytes after a free-flow electrophoresis run. The vesicles in the left peak have the same electrophoretic mobility as intact erythrocytes.

acid on the outside. The right, much more slowly moving vesicle population has their sialic acid hidden on the inside. In contrast to outside-out vesicles they cannot adsorb, for example, influenza virus on their surface. These results demonstrate the usefulness of electrophoresis for solving such problems.

A very promising and interesting example of the application of free-flow electrophoresis is in the isolation of DNA-envelope complexes of *E. coli* and *B. subtilis*. The success of these experiments was due to the additional electrical charge of the DNA units attached to the membrane fragments, which permits the separation of these complexes from the bulk of the envelope particles[28,29].

More examples of the application of the electrophoretic technique to the isolation of membrane systems could be given. In all of these, charges of functionally defined biological particles can be of decisive importance in difficult separation problems.

3.2. Analytical applications

3.2.1. Serum analysis

For the separation of serum proteins by analytical free-flow electrophoresis, a Tris–borate buffer of pH 8.8 was used[3].

Fig. 3 shows the results obtained from normal serum with separation times of 20, 28, 50 and 70 sec. A field strength of 140 V/cm was applied. The total amount of sample injected was 0.1 μl, corresponding 3 μg of protein (picomole range). The protein bands were detected photometrically in the absorption range of the peptide bond (225 nm). A separation of the serum proteins that could be quantitatively evaluated was possible after a separation time of only about 30 sec. Up to 100 samples can be tested per hour. The standard deviation of the reproducibility was proved by separating a normal serum 10 times. The averaged percentage values and the coefficients of variation (CVs) are indicated in Table 1 (column I) and compared with the CVs of membrane electrophoresis[30] (column II) and moving-boundary electrophoresis[31] (column III) taken from the literature. The results show that the analytical free-flow electrophoresis gives a good reproducibility.

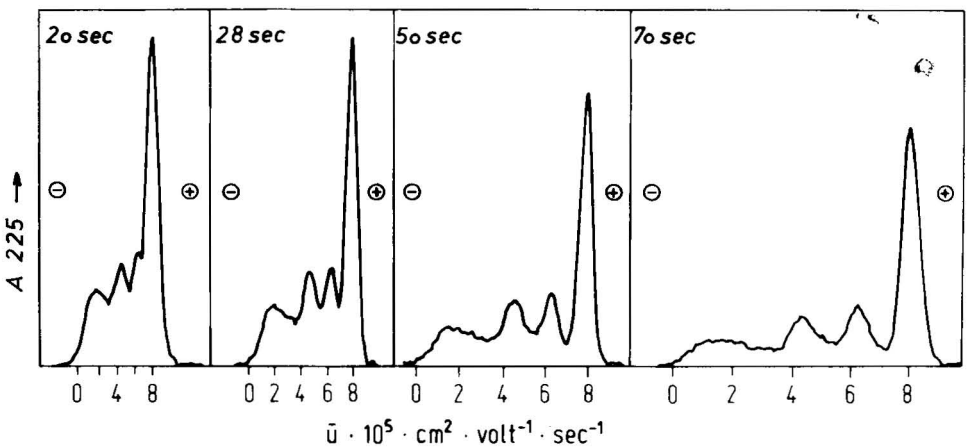

Fig. 3. Separation of serum proteins[3]. Tris–borate buffer (pH 8.8); 480 V, 42 mA.

TABLE 1

COMPARISON OF THE REPRODUCIBILITIES OF 10 SEPARATIONS OF A NORMAL HUMAN SERUM

I = Free-flow electrophoresis; II = membrane electrophoresis[30]; III = moving-boundary electrophoresis[31].

Component	*I*		*II*	*III*
	%	*CV*	*CV*	*CV*
Alb ($+\alpha_1$)	62.1	1.1	3.4	2.0
α_2	7.5	1.9	9.0	4.1
β_{1-3}	9.3	1.7	9.5	4.9
γ_{1-2}	21.2	3.2	9.4	5.0

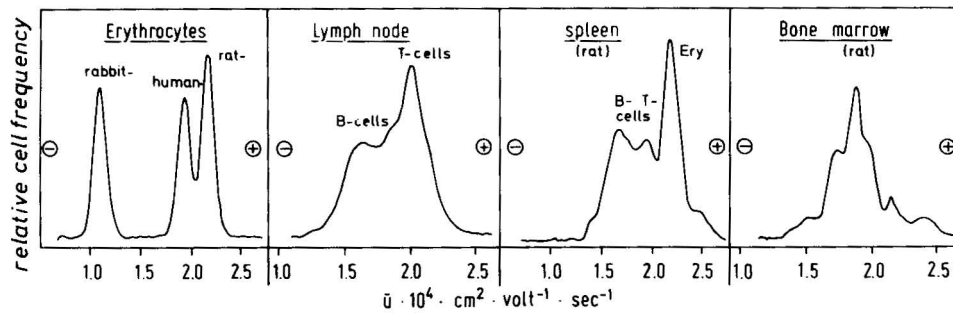

Fig. 4. Separation of diverse cell suspensions[3]. Triethanolamine buffer (pH 7.2); 470 V, 42 mA, residence time 40 sec.

3.2.2. Electrophoretic mobility measurements on cells

Fig. 4 shows separations of different cell materials. For one measurement, only 100,000 cells are required. The distribution pattern of the separated cell populations was achieved by means of nephelometry at 260 nm. In analytical free-flow electrophoresis, a large number of cells (17,000 per scan) is simultaneously monitored, implying a great statistical significance. From systematic experiments, an accuracy and reproducibility of mobility measurements of $\pm 1.4\%$ can be calculated[3].

As an example, an application of analytical free-flow electrophoresis in haematology is shown in Fig. 5. In the Tn syndrome, a rare condition of acquired mixed field polyagglutinability, some of the red cells have a lower sialic acid content and consequently a decreased electrophoretic mobility[32]. In the blood from a patient*, about 50% of the red cells are polyagglutinable erythrocytes, the others showing only a slight, unexplained decrease in electrophoretic mobility compared with normal human erythrocytes. Both cell populations could be separated successfully.

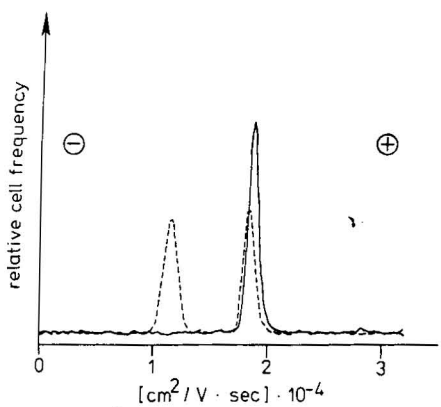

Fig. 5. Electrophoretic mobilities of native human erythrocytes from a patient with Tn syndrome (broken line) and normal native human erythrocytes (solid line). The low-mobility peak indicates the polyagglutinating erythrocyte population.

* The Tn blood sample was a gift from W. Dahr, Medizinische Universität, Köln, G.F.R.

We investigated the applicability of analytical free-flow electrophoresis to the macrophage electrophoretic mobility test for cancer diagnosis[33]. Further, the technique was used for the characterization of the electrophoretic mobilities of different postnatal erythrocyte populations of four different species (rat, sheep, mouse and man)[34].

3.2.3. Isoelectric focusing

In isoelectric focusing experiments, the long separation times required for proteins are incompatible with the principle and the advantages offered by the free-flow system as a fast separation method. The necessary stability is hardly maintained because of thermal convection in a slowly flowing buffer curtain. We avoided these difficulties to a large extent by modifying the separation chamber for the "recycling" procedure. For producing a pH gradient, the top and bottom are connected by means of 30 tubes, using the shortest route through a manifold peristaltic pump for buffer circulation. In this closed loop, an arbitrary number of separation cycles can be performed.

The example in Fig. 6 shows the isofocusing separation of sheep haemoglobin obtained in such a device. After 10 min of cycling, the pH gradient (1 % ampholine buffer, pH 3.5–10) was stabilized, the applied field strength being 140 V/cm. Then 20 μl of a 1 % haemoglobin solution were injected during 60 sec. After 30 cycles, *i.e.*, after an effective residence time of *ca.* 30 min, the final stage of focusing was reached. Continuation of the experiment did not improve the resolution further.

On the other hand, the separation of cells in a pH gradient show different properties. Biological membranes underly reversible and non-reversible changes in electrokinetic behaviour at non-physiological pH values and ionic strengths, owing to the denaturation of cell surface components and adsorption of hydrolysis products. The isoelectric point of cells, organelles or membranes is not a physical constant, as it is with proteins. Therefore, the importance and use of "cell isoelectric focusing" becomes doubtful. From very few experimental results, it can be concluded that neither during continuous operation nor in columns do native cells migrate in the pH gradient to their isoelectric points, which can theoretically be calculated on the basis of mobility measurements. The electrophoretic migration stops at a much

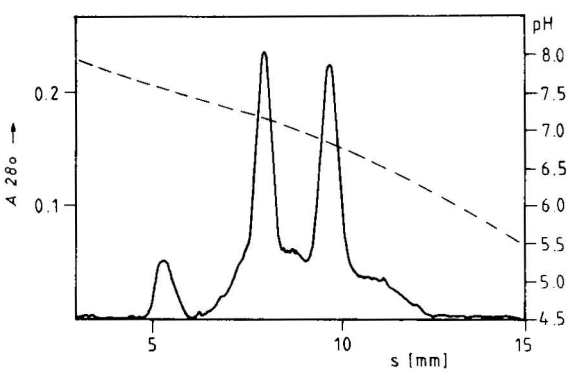

Fig. 6. Isoelectric focusing of sheep haemoglobin[3] with 1 % ampholine solution (pH 3.5–10); recycling method, 30 min.

higher pH level, which also depends on the environmental conditions (apparent isoelectric point), and the cell viability is usually completely lost[35].

4. SUMMARY

Several applications of preparative and analytical free-flow electrophoresis are described and discussed.

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STEADY-STATE RHEOELECTROLYSIS

HARRY RILBE

Department of Physical Chemistry, Chalmers Institute of Technology and University of Gothenburg, S-40220 Gothenburg (Sweden)

CONTENTS

1. INTRODUCTION

Isoelectric focusing in its modern form dates back to 1961[1], but it did not come into general use until Vesterberg[2] succeeded in synthesizing a useful system of carrier ampholytes and LKB started to supply commercially both columns and ampholytes, from then on called Ampholine[3]. The success of this method has been remarkable, and together with isotachophoresis the method has been the subject of four international symposia[4–7]. However, there are reasons for seeking improvements.

One reason is the expense of Ampholine, which certainly limits the use of isoelectric focusing, especially in preparative work in which large amounts are required. Another reason is the poor solubility of globulins in the isoelectric state in the absence of salts. Isoelectric focusing at an ionic strength much higher than that of focused Ampholine would certainly be able to keep many globulins in solution. A third reason is the insufficient knowledge of the milieu in which each individual protein is brought to rest in its isoelectric state. It is unsatisfactory that the protein in this state is surrounded by an unknown number of ampholytes, each with an unknown structure, conformation, molecular weight and concentration. It is especially unsatisfactory that the conductivity, and hence the field strength, generally assumes a very complicated course from anode to cathode, with several peaks and valleys. Conductivity courses have been recorded by Davies[8], but it should be borne in mind that the conductivity course during focusing is still more complicated as about half an hour of free diffusion during liquid transfer from column to conductolyser preceded Davies's records.

Catsimpoolas and co-workers, who have made considerable contributions to the development of isoelectric focusing, had a special reason to be concerned about the unpredictable conductivity course in isoelectric focusing and about its rapid changes after breaking the current. In a series of papers[9–12] they correctly pointed out that isoelectric focusing has possibilities in the measurement of diffusion coefficients and mobility slopes of focused proteins as these quantities dictate the zone width according to an equation given by Svensson (= Rilbe)[1] and that the rate of free diffusion can be measured by breaking the current. The great difficulties encountered in the interpretation of measurements in transient-state isoelectric focusing (TRANSIF) is intimately connected with the complicated behaviour of the conductivity in focusing, defocusing and refocusing. In a recent paper, Catsimpoolas[13] called for the synthesis of "second generation ampholytes", which hopefully will give not only a stable pH gradient, but also a uniform conductance and concentration distribution course throughout the separation path.

To summarize, there are reasons for requiring electrolytes and buffer systems that allow isoelectric focusing at higher ionic concentrations, that give rise to predictable and more easily controlled conductivity courses and that are cheaper than presently available carrier ampholytes.

Attempts to create stable pH gradients without the use of carrier ampholytes have already been reported. In 1970, Luner and Kolin[14] suggested the use of a temperature gradient as a means of obtaining a stable pH gradient. This method can be used only for very narrow pH regions as a pH span of 1 unit requires a temperature range of about 50 °K. It has not been worked out adequately and has not been taken up by other workers.

Another possibility for obtaining stable pH gradients in ordinary buffer solutions has been suggested by Troitsky *et al.*[15]. They pointed out that the influence of a reduced dielectric constant on the pK of a protolytic group is much less for proteins than for small ions as the sum of the ionic radii appears in the denominator of the appropriate equation. Thus, a concentration gradient of a non-electrolyte with a low dielectric constant in an aqueous buffer solution of constant composition must give rise to a stable pH gradient in which proteins can focus isoelectrically as their pI values are not much influenced by the varying dielectric constant. A pH range of 0.8–1.0 unit could be obtained with a glycerol gradient, and Troitsky *et al.* demonstrated focusing of haemoglobin and serum albumin in such gradients. This principle appears promising, but it has not been taken up by other workers.

Both the thermal and the dielectric pH gradients require large buffer reservoirs between the electrodes and the separation zone, otherwise acid and alkali will invade the latter and destroy the useful pH gradient. One important advantage of isoelectric focusing with Ampholine has thus been lost, namely the simplicity of an apparatus without electrode vessels.

Even before Vesterberg's[2] synthesis of Ampholine, we started to consider the possibility of creating stable pH gradients by steady-state electrolysis of ordinary buffer solutions in conjunction with superimposed liquid flows between various parts of the electrolysis cell. Such methods will subsequently be called steady-state rheoelectrolysis, in conformity with the Macheboeuf *et al.* term[16,17] "électrorhéophorèse" for paper electrophoresis with superimposed liquid flows. This method also involved a focusing effect giving considerable zone sharpening as the flow of water, which re-

placed evaporated water, increased towards the ends of the paper strips and completely balanced the electric migration.

In steady-state rheoelectrolysis, continuous exchange of liquid between the electrode compartments is especially simple to handle theoretically and will therefore be assumed to take place for the purposes of this paper, irrespective of the possibility that experimental practice may necessitate other arrangements.

2. PRINCIPLE OF OPERATION AND POSTULATES

The principle of rheoelectrolysis is shown schematically in Fig. 1. The electrolyser consists of one convection-free part (7) between the electrode compartments (2) and (5) with the electrodes (1) and (4). The anode (1) has to be made of platinum or carbon. The electrode compartments are constantly homogenized by the stirrers (3) and (6). The pumping device (8) produces a flow of anolyte through the ducts (9) and (10) to the cathode compartment, and a flow of catholyte through the ducts (11) and (12) to the anode compartment. Constant electric and hydrodynamic flows are allowed to continue until a steady state is reached.

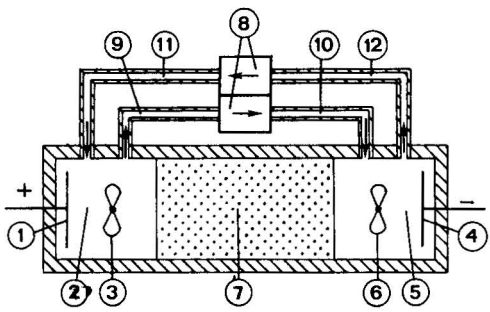

Fig. 1. Principle of steady-state rheoelectrolysis with liquid exchange between anolyte (2) and catholyte (5). The separation takes place in the convection-free portion (7), whereas anolyte and catholyte are constantly homogenized by the stirrers (3) and (6). The double pump (8) transfers equal volumes of liquid in both directions, and in the separation zone there should be no liquid flow.

By electrolysis without superimposed liquid flows, acids are drawn to the anode and bases to the cathode. Somewhere in the central part of the apparatus a complete evacuation of salts occurs and, if ampholytes are absent, a sharp change in pH from very low to very high values will be found at this point. With superimposed liquid flows as indicated in Fig. 1, acid will be transferred to the catholyte and base to the anolyte. If an ordinary buffer solution is being electrolysed, it is obviously possible in this way to retain pH values within the buffer range of the weak protolyte in both electrode vessels and thus to realize a smooth pH gradient within the same range in the convection-free part (7) of the electrolyser. It will be postulated that all electrolytes involved are resistant to anodic oxidation and cathodic reduction and that they suffer no losses due to evaporation or precipitation.

3. STEADY-STATE RHEOELECTROLYSIS WITHOUT INTERNAL LIQUID FLOW

If there is no internal liquid flow, the flow from the anode to the cathode is identical with that in the other direction. In the steady state, the liquid flows through

the pumps in Fig. 1 must balance exactly the added mass transports due to electric migration and diffusion for each ion constituent. This condition is expressed by the differential equation

$$\frac{Ti}{Fz} - Dq \cdot \frac{dC}{dx} = V(C_- - C_+) \tag{1}$$

where the first term on the left-hand side represents the electrical mass transport, the second term is the diffusional mass transport and the term on the right-hand side is the mass transport through the pumps. In eqn. 1, T is the transport number, C the concentration, D the diffusion coefficient, z the valence of the ion constituent in question, i the electric current, F the Faraday constant, q the cross-sectional area, x the coordinate from anode to cathode and V the volume flow-rate through the pumps.

This equation can be easily solved only if T and D are independent of x. As it is possible to choose experimental conditions that are likely to realize constant transport numbers and diffusion coefficients, this solution to the differential equation is of great interest. With constant T and D, dC/dx becomes constant and thus C is a linear function of x. The solution can be written as

$$C(x) - \bar{C} = \frac{Tix}{Fz\,Dq} - \frac{Vx(C_- - C_+)}{Dq} \tag{2}$$

where $\bar{C}$ is the mean concentration prevailing in the centre of the apparatus, where $x = 0$. The concentrations C_+ and C_- in anolyte and catholyte, respectively, are so far unknown, but can be eliminated by putting $x = a/2$, $C(x) = C_-$ and $x = -a/2$, $C(x) = C_+$, and taking the difference. The resulting equation can be solved for the concentration difference between catholyte and anolyte, which gives

$$C_- - C_+ = \frac{Tia}{Fz(Va + Dq)} \tag{3}$$

Insertion of eqn. 3 into eqn. 2 gives the final solution:

$$C(x) - \bar{C} = \frac{Tix}{Fz(Va + Dq)} \tag{4}$$

This equation has to be satisfied by every ion constituent present, but its validity is restricted to ion constituents that have constant transport numbers and diffusion coefficients throughout the electrolyser. In the following discussion, the treatment will be limited to buffers with only two ion constituents, both monovalent. Denoting the cation constituent by the subscript 1 and the anion constituent by the subscript 2, we thus obtain the two concentration courses:

$$C_1(x) = \bar{C}_1 + \frac{T_1 ix}{F(Va + D_1 q)} \tag{5}$$

and

$$C_2(x) = p\bar{C}_1 - \frac{T_2 ix}{F(Va + D_2 q)} \tag{6}$$

where $p\bar{C}_1$ replaces $\bar{C}_2$ and the dimensionless parameter p defines the composition of the original buffer solution:

$$p = \frac{\bar{C}_2}{\bar{C}_1} \tag{7}$$

3.1. Rheoelectrolysis of a buffer composed of a weak acid and its salt with a strong base

Constant transport numbers and diffusion coefficients cannot be expected unless both buffer components (salt and excess weak acid) are present everywhere in the apparatus. Consequently, we require $C_1(x)$ to be positive everywhere, even at the anode, and $C_2(x)$ to be larger than $C_1(x)$ everywhere, even at the cathode. These two conditions lead to the inequalities

$$2F\bar{C}_1 (Va + D_1 q) > T_1 ia \tag{8}$$

and

$$2F(p-1)\bar{C}_1 > i\left(\frac{T_1 a}{Va + D_1 q} + \frac{T_2 a}{Va + D_2 q}\right) \tag{9}$$

Both conditions have to be satisfied, and consequently the most restrictive one should be chosen. However, this depends on the starting conditions, and in order to avoid the mathematical inconvenience connected with subdivision of the treatment into two parallel sections, it is worth investigating the sort of starting conditions that make the two inequalities 8 and 9 identical. This can be done by solving the corresponding equations for i and putting the resulting expressions equal. This leads to the equation

$$p = 2 + \frac{T_2 (Va + D_1 q)}{T_1 (Va + D_2 q)} \tag{10}$$

With a knowledge of the two transport numbers and diffusion coefficients, it is possible to choose an excess of weak acid satisfying this equation. By doing this, rheoelectrolysis has the simplest possible mathematics, as will be shown below.

With the aid of eqn. 10, it is possible to eliminate $Va + D_2 q$ in favour of $Va + D_1 q$ in eqn. 6, which allows the deduction of the following simple expression for the excess of weak acid:

$$C_2(x) - C_1(x) = (p-1)\left[\bar{C}_1 - \frac{T_1 ix}{F(Va + D_1 q)}\right] \tag{11}$$

Finally, if the inequality 8 is replaced with the equation

$$T_1 ia = 2kF\bar{C}_1 (Va + D_1 q) \tag{12}$$

where k is a dimensionless parameter smaller than unity, this equation can be used for elimination of current, transport numbers and diffusion coefficients. Eqns. 5 and 11 then take the forms

$$C_1(x) = \bar{C}_1 (1 + 2kx/a) \tag{13}$$

and

$$C_2(x) - C_1(x) = (p-1)\bar{C}_1 (1 - 2kx/a) \tag{14}$$

These equations are very convenient for deduction of the pH course through the Henderson equation:

$$\mathrm{pH} = \mathrm{p}K - \log\left[\frac{C_2(x) - C_1(x)}{C_1(x)}\right] \tag{15}$$

Insertion of eqns. 13 and 14 gives

$$\mathrm{pH} = \mathrm{p}K - \log(p - 1) - \log\left(\frac{1 - 2kx/a}{1 + 2kx/a}\right) \tag{16}$$

By putting in succession $x = a/2$ and $x = -a/2$, one obtains the total pH range from anode to cathode:

$$\Delta\mathrm{pH} = 2\log\left(\frac{1 + k}{1 - k}\right) \tag{17}$$

Probably the value of k should not exceed 0.9 because a safety margin below the critical value of unity is advisable. One therefore concludes that it is possible to cover a pH range of about 2.6 units by rheoelectrolysis of a simple buffer solution. The minimal pH gradient is found in the centre of the apparatus, where it has the value

$$\frac{\mathrm{d}(\mathrm{pH})}{\mathrm{d}x} = 4\,(k/a)\log\mathrm{e} \tag{18}$$

The pH course given by eqn. 16 is illustrated in Fig. 2 for some different k values.

3.2. Rheoelectrolysis of a buffer composed of a weak base and its salt with a strong acid

This case is the converse of that just treated, and the requirements to be formulated are that $C_2(x)$ be positive for $x = a/2$ and that $C_1(x) - C_2(x)$ be positive for $x = -a/2$. These conditions lead to the inequalities

$$2pF\bar{C}_1\,(Va + D_2q) > T_2ia \tag{19}$$

and

$$2\,(1 - p)\,F\bar{C}_1 > ia\left(\frac{T_1}{Va + D_1q} + \frac{T_2}{Va + D_2q}\right) \tag{20}$$

Both of these conditions have to be satisfied, and therefore the simplest mathematics are obtained if they are identical. This occurs for

$$p^{-1} = 2 + \frac{T_1\,(Va + D_2q)}{T_2\,(Va + D_1q)} \tag{21}$$

which is to be compared with eqn. 10. Eqn. 21 can be used for elimination of $Va + D_1q$ in favour of $Va + D_2q$ in eqn. 5, which allows deduction of the following expression for the excess of weak base:

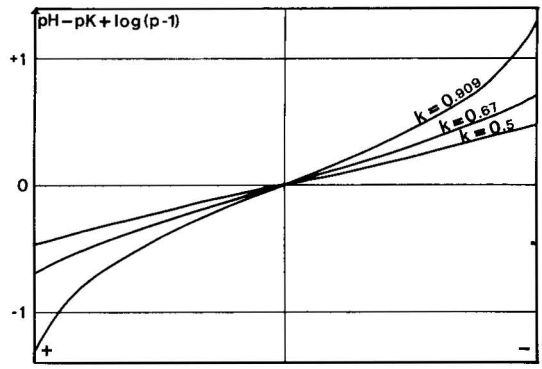

Fig. 2. Theoretical pH courses according to eqn. 16 for rheoelectrolysis of a buffer solution composed of a weak acid and its salt with a strong base. The liquid flow parameter, k, has an upper limit of unity.

$$C_1(x) - C_2(x) = (p^{-1} - 1)\left[\bar{C}_2 + \frac{T_2 ix}{F(Va + D_2 q)}\right] \tag{22}$$

Finally, if the inequality 19 is replaced by the equation

$$T_2 ia = 2kpF\bar{C}_1 (Va + D_2 q) \tag{23}$$

where $1 > k > 0$ as before, this equation can be used for elimination of transport numbers and diffusion coefficients. Eqns. 6 and 22 then take the forms

$$C_2(x) = \bar{C}_2 (1 - 2kx/a) \tag{24}$$

and

$$C_1(x) - C_2(x) = (p^{-1} - 1)\bar{C}_2 (1 + 2kx/a) \tag{25}$$

These equations should be compared with eqns. 13 and 14. Insertion of eqns. 24 and 25 into the Henderson equation for the present case:

$$\mathrm{pH} = \mathrm{p}K + \log\left[\frac{C_1(x) - C_2(x)}{C_2(x)}\right] \tag{26}$$

gives the following pH course for a buffer with a weak base:

$$\mathrm{pH} = \mathrm{p}K + \log(p^{-1} - 1) + \log\left(\frac{1 + 2kx/a}{1 - 2kx/a}\right) \tag{27}$$

which corresponds to the pH course given by eqn. 16.

It should be noted that the pH course in both types of buffer is not symmetrical about the pK of the weak acid or weak base. The point of symmetry in the centre of the electrolyser has a pH more remote from the neutral point. This pH displacement away from neutrality is $\log(p - 1)$ for a weak acid and $\log(p^{-1} - 1)$ for a weak base buffer.

3.3. Rheoelectrolysis of a buffer containing a salt of a weak acid and a weak base

Eqns. 5 and 6 are valid, and the requirements to be formulated are for $C_1(x)$ to be positive in the anolyte and for $C_2(x)$ to be positive in the catholyte. These conditions are

$$2F\bar{C}_1 (Va + D_1q) > T_1ia \tag{28}$$

and

$$2pF\bar{C}_1 (Va + D_2q) > T_2ia \tag{29}$$

They are identical for the p value

$$p = \frac{T_2 (Va + D_1q)}{T_1 (Va + D_2q)} \tag{30}$$

With a knowledge of the relevant data for the two ion constituents, it is possible to choose that buffer composition. If this is done, eqn. 6 can be written in the form

$$C_2(x) = p \left[\bar{C}_1 - \frac{T_1ix}{F(Va + D_1q)}\right] \tag{31}$$

The inequality 28 is further replaced with the equation

$$T_1ia = 2kF\bar{C}_1 (Va + D_1q) \tag{32}$$

where $1 > k > 0$. Insertion of i from this equation into eqns. 5 and 31 yields

$$C_1(x) = \bar{C}_1 (1 + 2kx/a) \tag{33}$$

and

$$C_2(x) = \bar{C}_2 (1 - 2kx/a) \tag{34}$$

Even in this case both concentration courses become linear. The neutral salt prevails where $C_1(x) = C_2(x)$, which occurs at the point

$$\frac{x}{a} = \frac{p - 1}{2k(p + 1)} \tag{35}$$

On the cathodic side of this point, the weak base is in excess, and consequently there is a buffer there with a pH course within the buffer range of the weak base. On the anodic side, the weak acid is in excess, and there we have a buffer with a pH course within the buffer range of the weak acid. Sufficiently far from the point given by eqn. 35, the Henderson equation can be used for calculation of these pH courses, eqns. 26 and 15, respectively, being applicable. In the vicinity of the point given by eqn. 35, however, the Henderson equation cannot be used because in this pH range both ion constituents are incompletely ionized. A correct treatment thus has to deal with dissociation

theory for two simultaneously present weak protolytes, which is too lengthy to be given here. The resulting expression for the pH course is complicated and will not be given here, but its graphical representation is presented in Fig. 3, valid for $p = 1$. As can be seen, the pH gradient has one minimum in each buffer range and a maximum at the location of the pure salt. The total pH range obtainable is greater than twice the pK difference between weak base and weak acid, but the pH course at the location of the pure salt becomes very steep for large values of ΔpK.

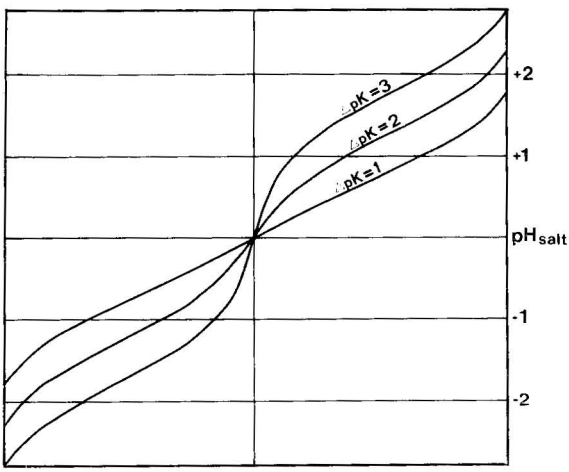

Fig. 3. Theoretical pH courses for rheoelectrolysis of a solution of a salt of a weak base and a weak acid for a liquid flow parameter $k = 0.9$ and for three different values of ΔpK between weak base and weak acid.

4. EXPERIMENTAL

Some attempts at experimental verification of the theorv have been made with various forms of multi-compartment apparatus. No protein separations have been performed with this new technique because so far all efforts have been devoted to creation of stable pH gradients with the use of ordinary buffer solutions.

The first attempts were unsuccessful. Thus, one experiment with tris lactate gave the pH course shown in Fig. 4, which has no great resemblance to the theoretical ones in Fig. 3 and is virtually useless. The reason for the large pH plateau at pH 4 was later traced to the fact that the two pumps in Fig. 1 gave unequal rates of flow, resulting in an internal liquid flow from anode to cathode. This will be dealt with in the next section.

After the introduction of arrangements to prevent internal liquid flow, the pH courses shown in Figs. 5 and 6 were obtained, the former with a sodium acetate buffer and the latter with a sodium borate buffer. The pH course in Fig. 5 should be useful although it does not have the fine symmetry possessed by the theoretical curves in Fig. 2. Fig. 5 also includes the conductivity course throughout the electrolyser. The pH course in the borate experiment (Fig. 6) is very good and agrees well with the theoretical shape shown in Fig. 2.

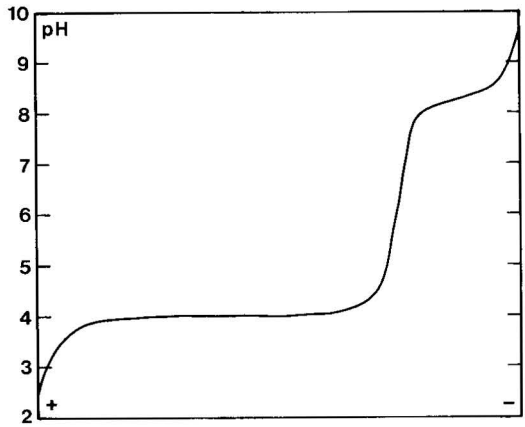

Fig. 4. Experimental pH course on rheoelectrolysis of tris lactate. There is no resemblance to the curves in Fig. 3 owing to an unintentional internal liquid flow from anode to cathode.

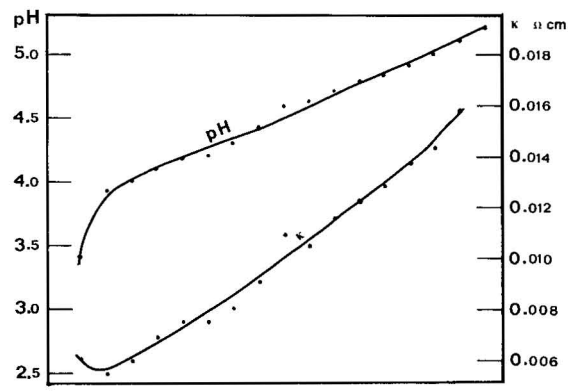

Fig. 5. Experimental pH and conductivity courses on rheoelectrolysis of an acetate buffer solution.

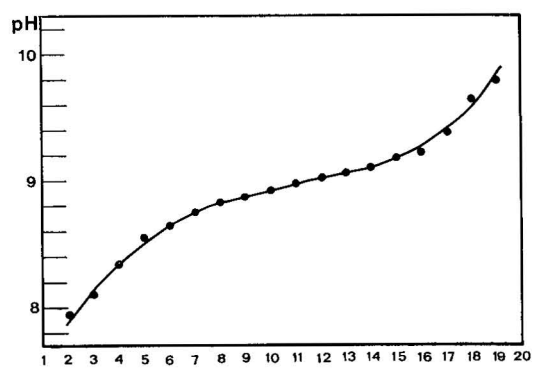

Fig. 6. Experimental pH course on rheoelectrolysis of a borate buffer solution.

5. STEADY-STATE RHEOELECTROLYSIS WITH INTERNAL LIQUID FLOW

If the flow through the cathodic pump is $V + v$ and that through the anodic pump is $V - v$, then the difference, $2v$, gives rise to an internal liquid flow from anode to cathode. The differential equation expressing the balance between the various mass transports then assumes the form

$$\frac{Ti}{Fz} - Dq \cdot \frac{dC}{dx} + 2vC = C_- (V + v) - C_+ (V - v) \tag{36}$$

The solution of this equation is too tedious to be deduced here, and for the present purpose it is sufficient to present the result:

$$C(x) = \bar{C} + \frac{Tia}{Fz} \cdot \frac{2va\, e^{2vx/Dq} - Dq\,(e^{2va/Dq} - 1)}{(2va)^2 + 2v\,(V + v)\,a^2\,(e^{2va/Dq} - 1)} \tag{37}$$

Owing to the presence of the term $2vC$ in eqn. 36, this concentration course is no longer linear, but exponential. Typical concentration courses according to eqn. 37 for cations and anions are shown in Fig. 7. Extended plateau concentrations as illustrated there are obtained when the internal liquid flow is considerable. From the exponential concentration functions, the pH course can also be calculated. The result of such a calculation is demonstrated in Fig. 8, in which eqn. 37 has been applied to rheoelectrolysis of tris lactate. The resemblance between Figs. 4 and 8 is obvious, and thereby it has been proved that the bad result in the tris lactate experiment was due to internal cathodic liquid flow. There is, however, one striking difference between the theoretical curve in Fig. 8 and the experimental curve in Fig. 4, *viz.*, the sharp pH decline in the latter close to the anode. This is explained by the fact that the base tris, contrary to the theoretical postulate, is destroyed by anodic oxidation.

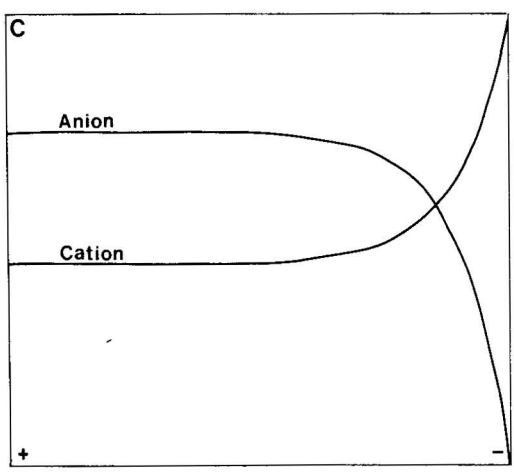

Fig. 7. Theoretical concentration courses for cation and anion according to eqn. 37 on rheoelectrolysis with a considerable internal liquid flow from anode to cathode.

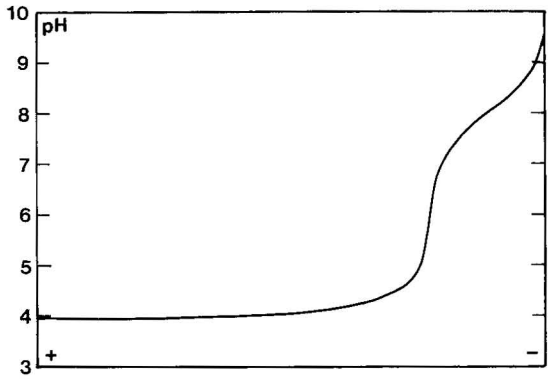

Fig. 8. Theoretical pH course for rheoelectrolysis of tris lactate with a considerable internal liquid flow from anode to cathode. Note the similarity in shape to the experimental curve in Fig. 4. The sharp decline in pH at the anode in Fig. 4 is due to anodic oxidation of tris, not taken into account in the theory behind eqn. 37.

6. DISCUSSION AND CONCLUSIONS

As is evident from the above discussion, this project has not advanced very far experimentally. Owing to a lack of personnel, it has not been possible until recently to begin to study the experimental problems inherent in the project. Various technical difficulties have proved to be more severe than was expected, and the effect of unavoidable small variations in transport numbers and diffusion coefficients have to be investigated both theoretically and practically. At present we are convinced that steady-state rheoelectrolysis is impossible in density gradients and that gels offer special complications not covered by the theory, as they do in conventional isoelectric focusing. Probably, therefore, analytical isoelectric focusing will continue to be dependent on Ampholine or other carrier ampholytes. Preparative work with multi-compartment types of apparatus appears at present to be the most promising application of steady-state rheoelectrolysis, but its degree of utility and versatility is still unknown.

7. ACKNOWLEDGEMENTS

The successful borate experiment was carried out at the Karolinska Institute in Stockholm by Mr. A. Forchheimer and the acetate experiment at this Institute by Dr. M. Almgren, both several years ago. The rheoelectrolysis project is at present financially supported by the Swedish Board for Technical Development, which is gratefully acknowledged.

8. SUMMARY

Rheoelectrolysis is defined as electrolysis of an electrolyte solution within a convection-free portion under simultaneous liquid exchange between two homogenized portions on either side of the convection-free portion of the electrolysis cell. As a special case, the two homogenized portions can be the anolyte and the catholyte, and

this case is particularly simple to treat theoretically. If electric and liquid flows are allowed to proceed at a constant rate for a sufficiently long time, a steady state develops that is characterized by a stable pH gradient in which isoelectric focusing can be carried out, provided that the electrolytes have suitable buffer properties. The tendency of the electric currrent to separate acids and bases completely is counteracted by pumps which transfer a more acidic solution on the anodic side to the cathodic side and a less acidic solution on the cathodic side to the anodic side of the convection-free portion of the electrolyser. In this way the two ion constituents of an ordinary buffer are made to circulate between the two homogenized portions as they migrate electrically within the convection-free portion and are pumped in the other direction outside the electrolyser.

If the transport numbers and diffusion coefficients of the ion constituents can be treated as constants, the differential equation of steady-state rheoelectrolysis is easily solved. If the two liquid flow-rates are identical, the concentration courses of the ion constituents become linear, and the pH course has a sigmoid shape. At least one of the ion constituents must be a weak protolyte, but if both have buffer action, the pH region that can be covered becomes much greater. The pH gradient decreases as the pumping speed increases.

If there is a net liquid flow within the convection-free portion of the electrolyser, the concentration courses of the ion constituents assume an exponential form, leading to a pH gradient that increases in the direction of this internal flow. Such gradients are not useful for isoelectric focusing.

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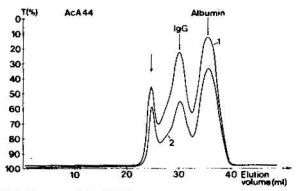

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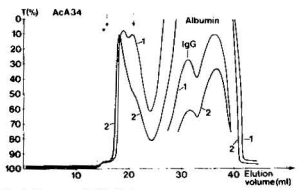

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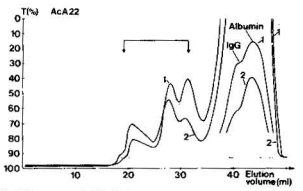

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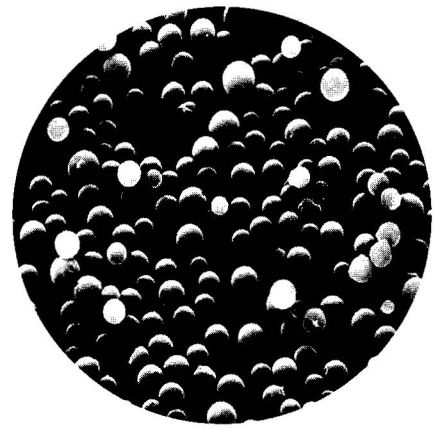

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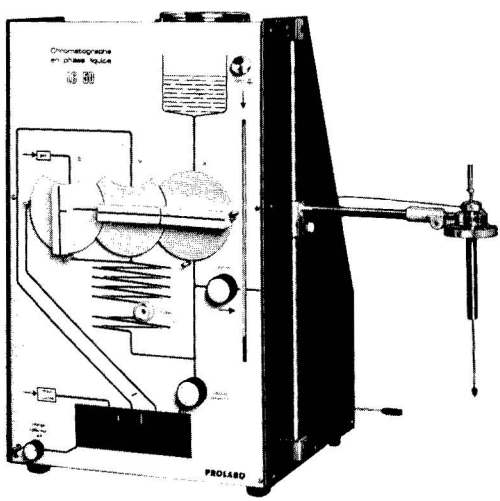

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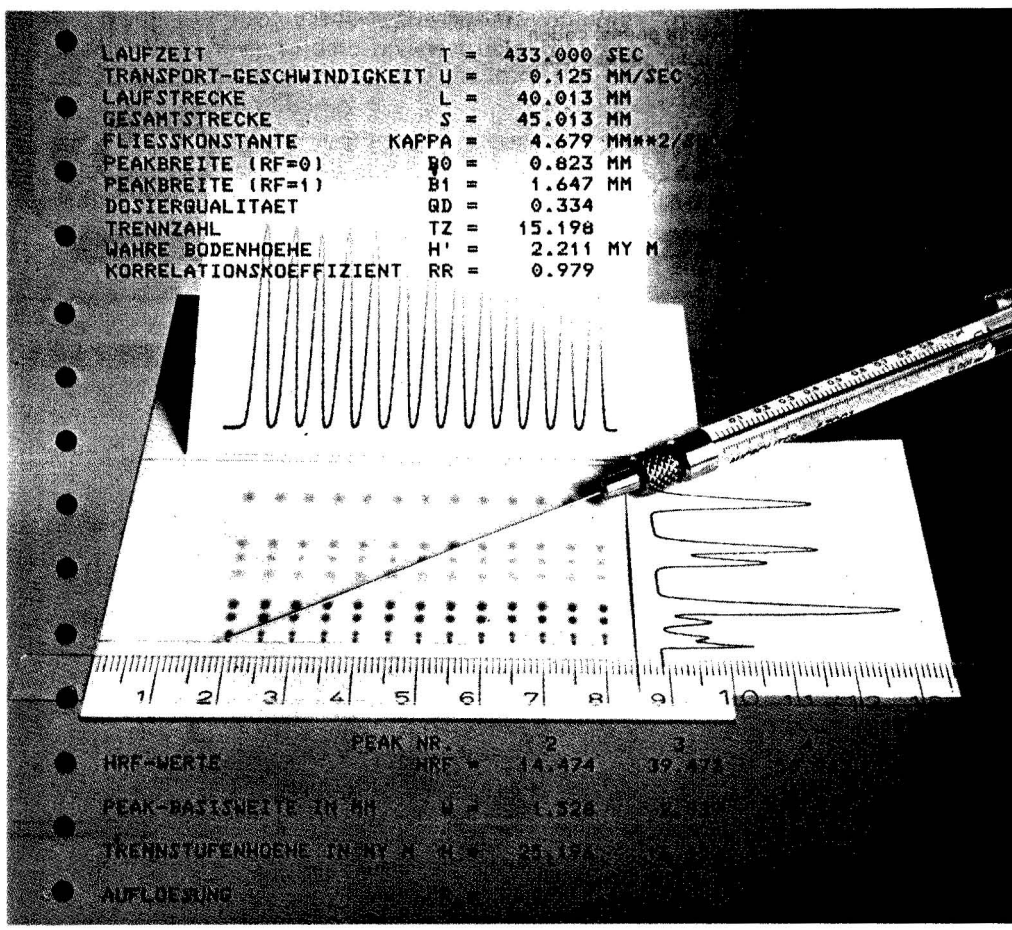

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Types of Contributions. The following types of papers are published in the *Journal of Chromatography* and the section on *Biomedical Applications:* Regular research papers (full-length papers), short communications and notes. Short communications are preliminary announcements of important new developments and will, whenever possible, be published with maximum speed. Notes are usually descriptions of short investigations and reflect the same quality of research as full-length papers, but should preferably not exceed four printed pages. For reviews, see page 2 of cover under Submission of Papers.

Manuscripts. Manuscripts should be typed in double spacing on consecutively numbered pages of uniform size. The manuscript should be preceded by a sheet of manuscript paper carrying the title of the paper and the name and full postal address of the person to whom the proofs are to be sent. Authors of papers in French or German are requested to supply an English translation of the title of the paper. As a rule, papers should be divided into sections, headed by a caption (e.g., Summary, Introduction, Experimental, Results, Discussion, etc.). All illustrations, photographs, tables, etc. should be on separate sheets.

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Summary. Full-length papers and review articles should have a summary of 50–100 words which clearly and briefly indicates what is new, different and significant. In the case of French or German articles an additional summary in English, headed by an English translation of the title, should also be provided. (Short communications and Notes are published without a summary.)

Illustrations. The figures should be submitted in a form suitable for reproduction, drawn in Indian ink on drawing or tracing paper. One original and two photocopies are required. Attention should be given to any lettering (which should be kept to a minimum) and to spacing on axes of graphs in order to ensure that numbers etc. remain legible after reduction. Axes of a graph should be clearly labelled. The figures should preferably be of such a size that the same degree of reduction can be applied to all of them. Photographs should have good contrast and intensity. Sharp, glossy photographs are required to obtain good halftones. References to the illustrations should be included in appropriate places in the text using arabic numerals. Each illustration should have a legend, all the *legends* being typed (with double spacing) together on a *separate sheet*. If structures are given in the text, the original drawings should be supplied. Coloured illustrations are reproduced at the authors' expense, the cost being determined by the number of pages and by the number of colours needed. The written permission of the author and publisher must be obtained for the use of any figure already published. Its source must be indicated in the legend.

References. References should be numbered in the order in which they are cited in the text, and listed in numerical sequence on a separate sheet at the end of the article. The numbers should appear in the text at the appropriate places in square brackets. In the reference list, periodicals [1], books [2], multi-author books [3] and proceedings [4] should be cited in accordance with the following examples:

1 A. T. James and A. J. P. Martin, *Biochem. J.*, 50 (1952) 679.
2 L. R. Snyder, *Principles of Adsorption Chromatography*, Marcel Dekker, New York, 1968, p. 201.
3 H. C. S. Wood and R. Wrigglesworth, in S. Coffey (Editor), *Rodd's Chemistry of Carbon Compounds, Vol. IV, Heterocyclic Compounds*, Part B, Elsevier, Amsterdam, Oxford, New York, 2nd ed., 1977, Ch. 11, p. 201.
4 E. C. Horning, J.-P. Thenot and M. G. Horning, in A. P. De Leenheer and R. R. Roncucci (Editors), *Proc. 1st Int. Symp. Quantitative Mass Spectrometry in Life Sciences, Ghent, June 16–18, 1976*, Elsevier, Amsterdam, Oxford, New York, 1977, p. 1.

Abbreviations for the titles of journals should follow the system used by *Chemical Abstracts*. Articles not yet published should be given as "in press", "submitted for publication", "in preparation" or "personal communication". The *Journal of Chromatography; Journal of Chromatography, Biomedical Applications* and *Chromatographic Reviews* should be cited as *J. Chromatogr.*

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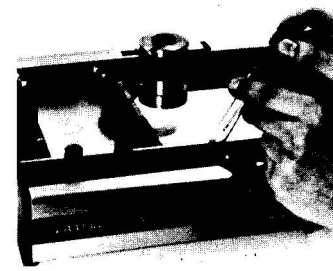
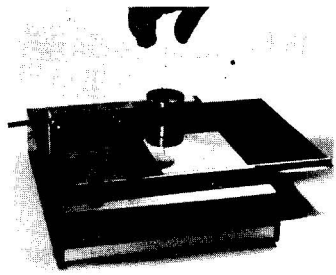
Fill capillary pipette

In the case of ordinary TLC plates this may be a glass capillary; for HP-TLC plates it should be a Pt-Ir pointed application capillary or a 100 or 200 nl Nanodoser.



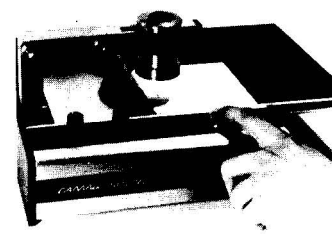


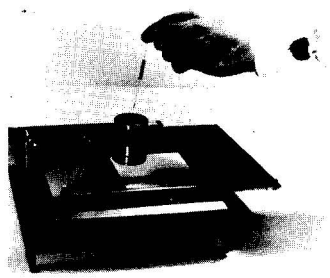
Place capillary pipette in the opening of the magnetic applicator head and let go

The capillary remains suspended approx. 10 mm above the layer.

Push release button

The capillary lowers at a preset speed down onto the layer, discharges and returns to its suspended position after a preset time,





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