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Review

Progress in affinophoresis

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CONTENTS

1. INTRODUCTION

Coupling of an affinity ligand to a soluble polyionic polymer such as succinylpolylysine makes it a specific mobile carrier in an electric field for substances having affinity to the ligand. The conjugate has a high electrophoretic mobility with a large electrokinetic force and therefore specifically changes the electrophoretic mobility of a substance that associates with the ligand. The conjugate is called an affinophore and the technique of electrophoresis using an affinophore is called affinophoresis[1] (Fig. 1).

The usefulness of biospecific affinity in the separation of biological molecules has become widely recognized since the development of affinity chromatography[2]. In

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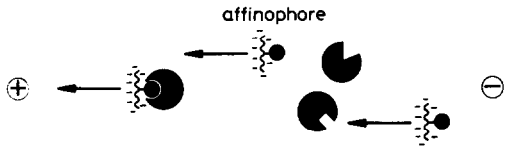

Fig. 1. The principle of affinophoresis.

the field of electrophoresis also, immobilization of affinity ligands on agarose or polyacrylamide gel has opened up new horizons for the separation of proteins and the determination of biospecific affinity[3–5]. However, the feasibility of an alternative type of matrix, the "mobile" matrix, had not been realized until the development of affinophoresis.

The key to the successful application of biospecific affinity to the separation of biological macromolecules or to biochemical analyses is the use of good matrices devoid of non-specific binding. From this point of view, the use of polyionic polymers as affinity matrices seemed dubious, since ionic polymers could be regarded as "spiders' webs" catching all substances having opposite charges. The success of affinophoresis, however, showed that there is ample scope for the use of polyionic polymers as mobile affinity matrices.

The use of mobile matrices gives affinophoresis several unique features. First, it is applicable to substances which have little or even no electrophoretic mobility, such as a protein at its isoelectric point. Second, it is applicable to suspended particles such as cells, because affinophoresis does not necessarily require an insoluble support.

In this review, selected results obtained with this distinctive method are presented and some of its basic aspects are discussed as a guide to its potential applications.

2. RELATED TECHNIQUES

Affinophoresis is a type of affinity electrophoresis, which is a method of analysis or separation of biological substances based on the change in electrophoretic mobility due to the formation of complexes with other substances by specific affinity. Affinity electrophoresis can be classified into four categories according to two criteria: whether both interacting substances are mobile (mobile system) or one of them is immobile (immobile system), and whether one of the interacting substances is linked or not to a matrix, either mobile or immobile (Table 1).

Cross-electrophoresis[6], which was used to detect protein–protein, protein–nucleic acid affinity, etc., is the root of affinity electrophoresis and is a representative of "mobile" affinity electrophoresis without matrices. The interaction between glycoproteins and lectins was determined by using this system[7]. Crossed immunoelectrophoresis[8] and gel shift assay for the analysis of nucleic acid–protein interactions[9] also come into this category, although they utilize changes in other parameters in addition to the pure electrophoretic mobility, *i.e.*, the solubility and the size of the complexes, respectively. When one of the interacting substances is inherently insoluble, the technique should be called "immobile" affinity electrophoresis without matrices. The electrophoresis of some plant lectins in starch gel[10] corresponds to this peculiar case.

TABLE 1

AFFINITY ELECTROPHORESIS CLASSIFIED ACCORDING TO TWO CRITERIA

From ref. 50.

Criterion	*Mobile system*	*Immobile system*
Without matrices	Cross-electrophoresis[6] Affinity electrophoresis of glycoproteins in the presence of lectins[7] Crossed immunoelectrophoresis[8] Gel shift assay[9] Antigen-specific electrophoretic cell separation[16]	Electrophoresis of lectins in starch gel[10]
With matrices	Affinophoresis[1,26,28,32,46,47,49,50] (mobile matrices: succinylpolylysine, polyacrylyl-β-alanyl-β-alanine, diethylaminoethyl-dextran)	Affinity electrophoresis with immobilized ligands[3–5,11–13] (immobile matrices: polyacrylamide gel, agarose gel)

Immobile matrices, such as polyacrylamide gel[3,5] or agarose gel[4], on which one of the interacting substances is coupled, were introduced together with the name "affinity electrophoresis" as the counterpart of affinity chromatography. "Immobile" affinity electrophoresis with matrices has been applied for quantitative studies of many affinity systems[11,12]. Separation of a polyclonal antibody into distinct monoclonal antibody species and the simultaneous determination of their affinity constants so far represents the summit of the achievements with this method[13]. Affinophoresis should be categorized as "mobile" affinity electrophoresis with matrices. Affinophoresis and affinity electrophoresis with immobilized ligands can thus be categorized as affinity electrophoresis with matrices.

Apart from the biospecific affinity, the micelle of dodecyl sulphate has also been successfully used as a mobile carrier in electrophoresis, *i.e.*, polyacrylamide gel electrophoresis of protein in the presence of the detergent[14] and electrokinetic chromatography for lipophilic molecules[15].

In order to separate cells based on the difference in surface antigens, a technique called antigen-specific electrophoretic cell separation (ASECS) has been developed[16]. In this method, the electrophoretic mobility of specific cells is decreased by treatments with homologous antibody and second antibody. ASECS could be classified as a type of mobile affinity electrophoresis without matrices.

3. AFFINOPHORESIS OF PROTEINS

3.1. Affinophores and affinophoresis

Affinophores have been prepared by using three types of matrices, *i.e.*, diethylaminoethyldextran, polyacrylyl-β-alanyl-β-alanine and succinylpolylysine. Examples of affinophoresis with these affinophores will provide a good introduction to the method. Affinophoresis can be carried out in free solution in principle, but the use of an insoluble gel support greatly facilitates its application, as in the case of ordinary

electrophoresis of proteins. Agarose gel of about 1% is porous enough for free electrophoresis of affinophore–protein complexes and can be easily impregnated with an affinophore by adding the affinophore to a warm solution of agarose before casting a gel.

3.1.1. Cationic affinophore based on dextran. The first affinophore prepared was a cationic type bearing an affinity ligand for trypsins[1]. Dextran was chosen for the base material because it has little tendency to bind proteins by non-specific interactions and because of the availability of size-fractionated polymers from commercial sources. The second point is important for free electrophoresis of an affinophore–protein complex in the support gel. For the derivatization of dextran, an alkylation reaction under conditions similar to those used in Hakomori's methylation methods for saccharides was utilized[17–19]. Dextran (average molecular weight 10 000) dissolved in dimethyl sulphoxide was treated with sodium methylsulphinyl carbanion and then alkylated with N-chloroacetyl-6-aminohexanoic acid and diethylaminoethyl bromide. The reaction introduced ω-carboxyl spacers for the subsequent coupling of affinity ligands and cationic charges of diethylaminoethyl groups onto the dextran. A competitive inhibitor for trypsin, *m*-aminobenzamidine[20], was coupled with the aid of a water-soluble carbodiimide [1-ethyl-3-(3-dimethylaminopropyl)carbodiimide][21] to the spacer. The affinophore consisted of dextran of about 60 glucose residues, carrying about 10 affinity ligands and about 25 positive charges (the sum of those of the ligands and the diethylaminoethyl groups) on average.

The affinophore was effective for the affinophoresis of trypsins from three different sources. At pH 7, the migration of cationic trypsins from bovine and *Streptomyces griseus*[22] was increased and that of anionic trypsin from *Streptomyces erythreus*[23] was decreased. As expected from the principle of affinophoresis, *Streptomyces erythreus* trypsin was carried towards the cathode at its isoelectric point, pH 4, in the presence of the cationic affinophore (Fig. 2). These effects of the affinophore were completely suppressed by adding a competitive inhibitor, leupeptin[24], to the gel or were not observed for trypsins irreversibly inhibited with tosyllysine chloromethyl ketone (TLCK)[25]. The mobility of chymotrypsin, which has a similar structure but differs in substrate specificity from trypsin, was not affected[26]. The results indicate that the alteration of the mobility depends on the specific affinity between the active site of trypsins and the affinity ligands on the affinophore.

The affinophore was deeply stained by acidic dyes generally used for the detection of proteins in support gels, such as Coomassie Brilliant Blue R250 or Amido Black 10B. After the affinophoresis, the gel was dehydrated with acetone and the proteins were detected by reaction of fluorescamine[27] with the amino groups of the proteins to form fluorescent derivatives. Another point to note is that this affinophore is slightly adsorbed by agarose gel. These aspects will be discussed below.

3.1.2. Anionic affinophore based on polyacrylic acid derivative. An affinophore can be either cationic or anionic. The second affinophore prepared was an anionic affinophore for trypsin[28]. Radical polymerization of acrylyl-β-alanyl-β-alanine provided an anionic affinophore matrix, in which carboxyl groups exist at the tips of hydrophilic spacers protruding from the polymer backbone. The polymerization reaction was carried out in the presence of thioglycolic acid to reduce the degree of polymerization. Although the degree of polymerization of the polymer has not been determined, gel chromatography of the polymer through a column of Sepharose

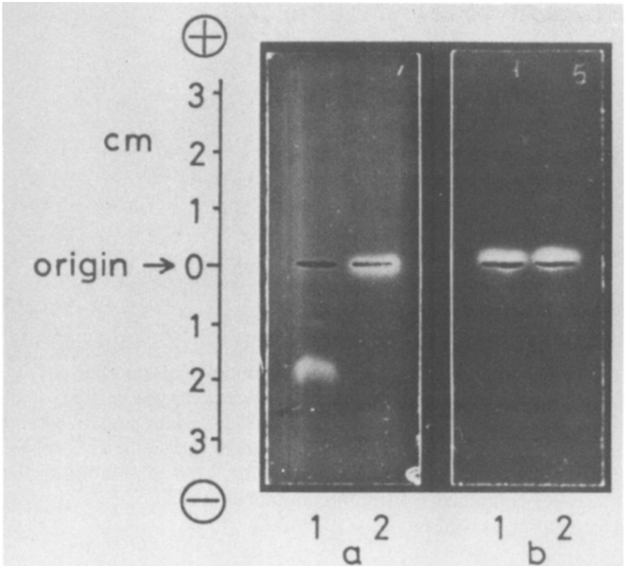

Fig. 2. Affinophoresis of *Streptomyces erythreus* trypsin at its p*I*. Electrophoresis of *Streptomyces erythreus* trypsin (4 μg, lane 1) and the trypsin inhibited with TLCK (4 μg, lane 2) was carried out for 1 h, at a constant current of 1.2 mA per plate in 0.1 *M* sodium acetate buffer (pH 4.0). Agarose gel plates (0.8%, 76 mm long × 26 mm wide × 0.65 mm thick) were prepared in (a) the presence or (b) the absence of the cationic benzamidine–affinophore. Proteins were detected after reaction with fluorescamine, as fluorescent bands. (From ref. 1.)

CL-6B indicated that the hydrodynamic volumes of most of the polymer molecules are considerably smaller than that of immunoglobulin G, which can move almost freely through 1% agarose gel. *p*-Aminobenzamidine was coupled to one-fifth of the carboxyl groups of the polymer by the use of water-soluble carbodiimide. This affinophore was also stained with Coomassie Blue dye, but it was no longer stainable after the change of the ionic groups from carboxyl to sulphonic acid, which was accomplished by the coupling of aminomethanesulphonic acid to the carboxyl groups by the use of the carbodiimide.

The mobilities of *S. griseus* and bovine trypsins were greatly changed in the presence of the anionic affinophore (Fig. 3). The dose–response relationship showed that *S. griseus* trypsin has a higher affinity for the affinophore than bovine trypsin does. This is consistent with the observation that the inhibition constant of benzamidine for *S. griseus* trypsin is 1.0 μM and that for bovine trypsin is 15 μM[29,30]. The slight anodic shift of TLCK-treated *S. griseus* trypsin and TLCK-treated bovine trypsin was once ascribed to a non-specific ionic effect of the polymer. However, this is not likely considering the high potency of the phosphate buffer used in the experiments for suppressing such ionic interactions (see below). Some other type of low-affinity interaction may be operating. Migration of *S. erythreus* trypsin, an anionic trypsin, was not influenced by the affinophore. Although the reason for this ineffectiveness was not studied, it seems possible that the ionic repulsion between the protein and the

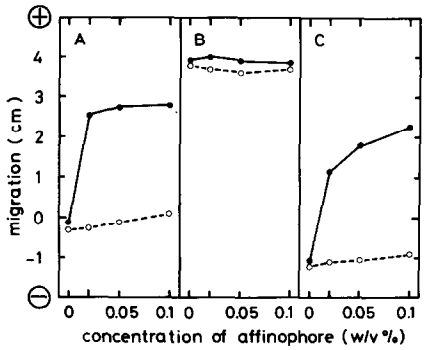

Fig. 3. Dependence of the migration of trypsins on the concentration of the anionic benzamidine–affinophore. Electrophoresis of (●) trypsins and (○) those inhibited with TLCK (4 μg each) was carried out at a constant current of 100 mA per plate for 40 min on an agarose gel plate (1%, 125 mm long × 80 mm wide × 1 mm thick) containing the indicated concentration of the affinophore and 0.1 *M* sodium phosphate buffer (pH 7). Proteins were detected by staining with Coomassie Brilliant Blue R250. A solution of the affinophore at a concentration of 0.1% (w/v) contained 0.46 m*M* *p*-aminobenzamidine. A, *S. griseus* trypsin; B, *S. erythreus* trypsin; C, bovine trypsin. (From ref. 28.)

affinophore interferes with the specific interaction. Alternatively, the difference in the mobilities of the protein and its complex with the affinophore might be too small. *S. griseus* trypsin in pronase, a mixture of proteases[31], was separated from other proteins by the affinophoresis (Fig. 4). In this way, the affinophoresis of trypsins was realized with either a cationic or an anionic affinophore.

3.1.3. Anionic affinophore based on polylysine. Although polyacrylyl-β-alanyl-β-alanine is an excellent matrix for an anionic affinophore, the synthesis of the monomer is laborious for biochemists who are interested in particular applications of affinophoresis, and the degree of polymerization cannot readily be determined. Poly-L-lysine is commercially available as a size-fractionated polymer. Although the polymer might be usable as a cationic matrix as it is, it was used after succinylation as an anionic matrix in order to avoid the problems associated with the use of a cationic affinophore, *i.e.*, adsorption on agarose gel and staining by dyes. Poly-L-lysine with an average degree of polymerization of 190 was succinylated and L-tryptophan methyl ester was coupled to one-fifth of the succinyl groups as the precursor of an affinity ligand for chymotrypsin and its derivatives[32]. This affinophore was also weakly stained with Coomassie Blue dye, but the coupling of aminomethanesulphonic acid to the residual carboxyl groups overcome this problem. Finally, the ester moiety of the ligand was removed by alkali treatment. The affinity ligand was tryptophan with a free carboxyl group and was thus a product-type ligand for chymotrypsin.

Two chemically modified derivatives of chymotrypsin were subjected to affinophoresis by using the tryptophan–affinophore together with chymotrypsin and chymotrypsinogen[32]. Phenylmethanesulphonylchymotrypsin (PMS-chymotrypsin) was produced by affinity labelling of the hydroxyl group of the active centre serine residue of chymotrypsin with phenylmethanesulphonyl fluoride (PMSF) and thus the substrate-binding pocket was occupied by the phenyl ring of the reagent[33]. Anhydro-chymotrypsin is a product of a β-elimination reaction of phenylmethanesulphonic acid

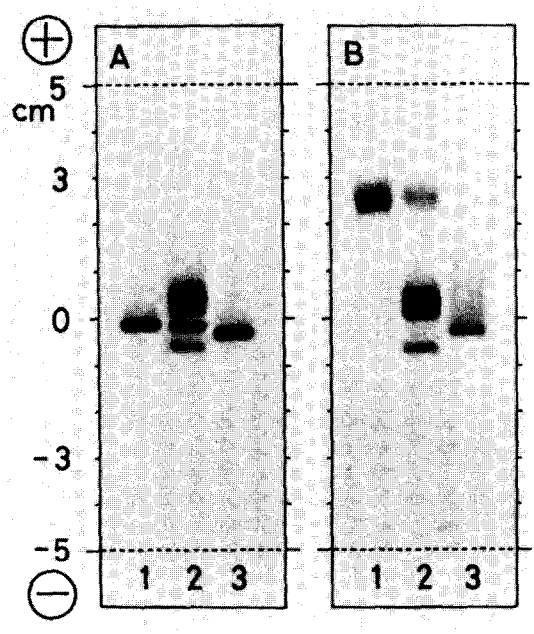

Fig. 4. Separation of *S. griseus* trypsin from pronase by affinophoresis. Electrophoresis of *S. griseus* trypsin (4 μg, lane 1), pronase (16 μg, lane 2) and the trypsin inhibited with TLCK (4 μg, lane 3) was carried out in (A) the absence or (B) the presence of the anionic benzamidine–affinophore (0.02%, w/v). Other conditions as in Fig. 3. Both sides (20 mm wide) of the gels were cut off and the central parts of the gel plates are shown. (From ref. 28.)

from PMS-chymotrypsin and has a dehydroalanine residue instead of serine at the active centre of chymotrypsin[34]. The transformation of chymotrypsin to its anhydro derivative enhances its affinity toward its own product-type ligands, *e.g.*, the affinity ligand of the tryptophan–affinophore[35,36]. The original mobilities of these proteins were very small with the electrophoresis buffer of 0.1 *M* sodium phosphate (pH 7.2) (Fig. 5). Addition of the affinophore to the gel greatly increased the mobility of anhydrochymotrypsin towards the anode and moderately increased that of chymotrypsin. For chymotrypsinogen and PMS-chymotrypsin, the effect of the affinophore was very small and it is consistent with the defectiveness of the substrate binding site of these proteins.

3.2. Some considerations on affinophoresis

Some discussion of the theoretical basis of affinophoresis would be helpful for the understanding of the method and useful for its further development.

3.2.1. Mobility. Suppose an affinophore (A) and a protein (P) are in the following equilibrium:

$$A + P \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} AP \qquad (1)$$

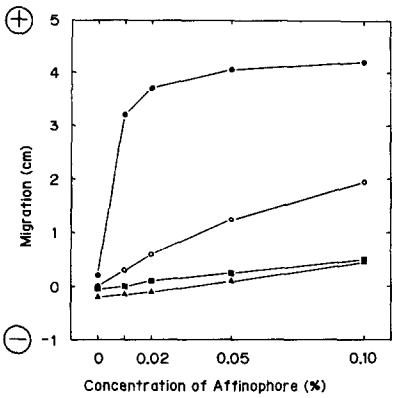

Fig. 5. Dependence of the migration of chymotrypsin and related proteins on the concentration of the tryptophan–affinophore. Electrophoresis of proteins (4 μg each) was carried out at a constant current of 100 mA per plate for 30 min on the agarose gel plate (1%, 125 mm long × 80 mm wide × 1 mm thick) containing the indicated concentration of the affinophore and 0.1 *M* sodium phosphate buffer (pH 7.2). Proteins were detected by staining with Coomassie Brilliant Blue R250. A solution of the affinophore at a concentration of 0.1% (w/v) (15 μ*M*) contained 0.56 m*M* tryptophan. (○) Chymotrypsin; (●) anhydrochymotrypsin; (■) PMS-chymotrypsin; (▲) chymotrypsinogen A. (From ref. 32.)

For an affinophore bearing a number of ligands, complexes of higher order, AP_2, AP_3, etc., should also be formed. For simplicity, it is assumed here that only the one-to-one AP complex is formed. This situation would be realized in the presence of an excess of affinophore over a protein. The change in the electrophoretic mobility in affinophoresis is based on the difference between the original mobility of the protein (m_0) and that of the AP complex (m_c). The mobility of a particle in an electrophoresis experiment is the velocity reached when the frictional drag just balances the electric force, *i.e.*, $QE = kv$, when a particle of charge Q migrates at velocity v in an electric field E with frictional constant k. On application of an electric field, the time required for a protein with a molecular weight of $1.2 \cdot 10^5$ to reach a constant velocity was calculated to be of the order of 10^{-13} min[37]. On the other hand, the rate constants of dissociation of enzyme–substrate complexes (k_{-1}), which would be comparable to that of the AP complex, are known to be 10^2–10^6 min^{-1} in general[38]. In other words, at the fastest, the dissociation reaction occurs once in 10^{-6} min for a single protein molecule under conditions that allow almost all the protein to form the complex with the affinophore. Hence the dissociation–association reaction is very slow compared with the time required for the change in electrophoretic velocity associated with the reaction. Consequently, the mobility of proteins subjected to affinophoresis is microscopically discontinuous, changing between m_0 and m_c in each dissociation and association reaction. The observed mobility of the protein (m) is an average as follows:

$$m = \frac{P_f}{P_t} \cdot m_0 + \frac{P_c}{P_t} \cdot m_c \tag{2}$$

where P_f and P_c are the concentration of the free protein and that of the protein complexed with the affinophore, respectively, and P_t is the total concentration of the

protein, *i.e.*, $P_t = P_f + P_c$. The equilibrium obeys the mass action law and the equilibrium constant (K_d) is given by

$$K_d = \frac{P_f[A]}{P_c} \tag{3}$$

where [A] is the concentration of the free affinophore. From eqns. 2 and 3, the following equation describing the change in mobility of a protein subjected to affinophoresis is obtained:

$$m - m_0 = (m_c - m_0)\frac{[A]}{K_d + [A]} \tag{4}$$

This equation is a rectangular hyperbola (Fig. 6) similar to the Henri–Michaelis–Menten equation of enzyme kinetics. The observed mobility of the protein (m) reaches that of the complex (m_c) at infinite concentration of the affinophore. Two important points concerning the effectiveness of affinophores are clearly shown. First, the difference between the original mobility of the protein and the mobility of the complex determines the maximum effect, but the mobility of the affinophore itself does not directly relate to the effectiveness. Second, the concentration of the affinophore should be comparable to or greater than the dissociation constant.

The double reciprocal plot of $1/(m - m_0)$ *versus* $1/[A]$ is linear and corresponds to the Lineweaver–Burk plot of enzyme kinetics:

$$\frac{1}{m - m_0} = \frac{K_d}{m_c - m_0} \cdot \frac{1}{[A]} + \frac{1}{m_c - m_0} \tag{5}$$

The intercept on the $1/(m - m_0)$ axis is $1/(m_c - m_0)$ and that on the $1/[A]$ axis is $-1/K_d$. An identical equation was derived for affinity electrophoresis through different approaches[39,40].

The results of the affinophoresis of chymotrypsin and anhydrochymotrypsin presented in Fig. 5 were plotted according to eqn. 5 (Fig. 7). Straight lines were fitted

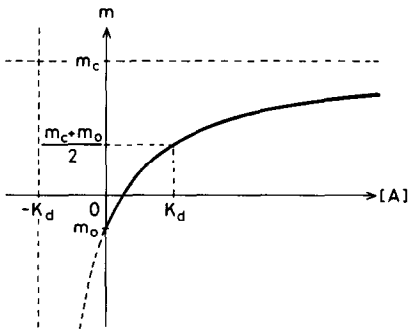

Fig. 6. Plot of m *versus* [A] according to eqn. 4; m is the observed mobility of the protein and [A] is the concentration of the affinophore.

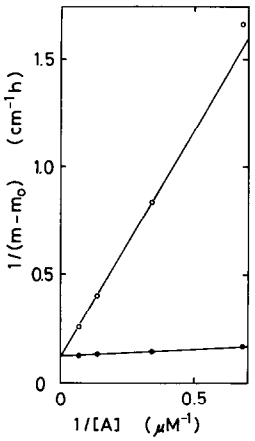

Fig. 7. Double reciprocal plot of the results of the affinophoresis of (○) chymotrypsin and (●) anhydrochymotrypsin according to eqn. 5.

and the values of $m_e - m_0$ and K_d were obtained as 8.8 cm h^{-1} and 18.1 μM for chymotrypsin and 8.4 cm h^{-1} and 0.60 μM for anhydrochymotrypsin, respectively (Fig. 7). In this case, the concentration of the affinophore was calculated simply by dividing the concentration of the lysine residue, which can be determined by amino acid analysis after hydrolysis, by the average degree of polymerization of the base polymer.

3.2.2. Number of ligands. At a rough estimate, the dissociation constant for an affinophore bearing n ligands [$K_d(n)$] should decrease from that for the affinophore bearing a single ligand [$K_d(1)$] by a factor of $1/n$, since there are n ways of complex formation, *i.e.*, $K_d(n) = K_d(1)/n$. The estimated number of ligands on the tryptophan–affinophore was 38. The value of $K_d(1)$ can then be calculated to be 690 μM for chymotrypsin and 23 μM for anhydrochymotrypsin by multiplying the K_d values obtained above by 38. The dissociation constants with acetyl-L-tryptophan at pH 6.0 (4°C) were reported to be 400 μM for chymotrypsin and 14 μM for anhydrochymotrypsin[35]. These values are roughly comparable to the $K_d(1)$ values calculated above. Hence increasing the number of ligands on an affinophore would be effective in raising its affinity for a protein.

For proteins having two or more binding sites for the ligand, the number of ligands on a single affinophore molecule would have another special effect. If each binding site of the protein can simultaneously bind to the ligands on such a flexible polymer, the interaction should be very strong, just like the avidity or bonus effect of an antibody–antigen interaction.

3.2.3. Size of affinophore. The electrophoretic mobility of a linear polyionic polymer such as polyacrylic acid is known to be independent of its degree of polymerization above a certain ionic strength[41]. The affinophore should be large enough to retain its high mobility on association with a protein. When an insoluble gel support for electrophoresis is used, its structure should be such as to allow unrestricted migration.

3.2.4. Heterogeneity of affinophore. Polymers used as the matrices in affino-

phoresis are heterogeneous in size, which would make the electrophoretic mobility of the AP complex heterogeneous. When an affinophore and protein are in equilibrium with rapid association–dissociation, each protein molecule undergoes many association–dissociation reactions with many different affinophore molecules in the course of affinophoresis. Thereby, the mobility of the AP complex approaches an average value, and thus the heterogeneity of the affinophore does not result in diffusion of protein bands in the direction of electrophoresis. If the value of k_{-1} is assumed to be 10^2 min^{-1}, a value which is low compared with k_{-1} proposed for usual enzyme–substrate complexes[38], an affinophoresis time of 30 min will allow a protein to bind about 3000 different affinophore molecules, and this number is sufficiently large for close approach to an average mobility.

For a binding equilibrium with extremely slow dissociation, *e.g.*, the biotin–avidin system in which k_{-1} is $2.4 \cdot 10^{-6}$ min^{-1} (ref. 42), heterogeneity of the affinophore might cause the diffusion of the protein bands in the direction of the electrophoresis.

3.2.5. Ionic interaction. It is desirable that the non-specific ionic interaction of proteins with an affinophore matrix should be as small as possible. To examine the ionic interaction between proteins and soluble polyionic polymers, electrophoresis of proteins was carried out on 1% agarose gel plates in the presence of succinylpolylysine (average degree of polymerization 120)[43]. Proteins migrated as distinct bands even in the presence of the polyionic polymer, but the mobility of cationic proteins was influenced. By using 0.1 *M* tris(hydroxymethyl)aminomethane (Tris)–acetic acid buffer (pH 7.9, ionic strength 0.06), the mobility of lysozyme (chicken), cytochrome *c* (horse) and chymotrypsinogen A (bovine) was greatly changed (about 80% of the maximum change) even at 10 μM polymer (corresponding to 1.2 m*M* lysine residue), whereas that of anionic proteins was not affected.

The relationship between the mobility of a protein and the concentration of the polymer was identical with that for affinophore–protein interaction, *i.e.*, it can be described as a rectangular hyperbola. If the formation of a distinct complex is assumed for the ionic interaction between cationic proteins and succinylpolylysine as in the case of specific interaction, the extent of ionic interaction can be estimated by using a dissociation constant as for affinophoresis. As expected, the ionic interaction is sensitive to the ionic strength of the electrophoresis buffer.

By using 0.1 *M* sodium phosphate buffer (pH 7, ionic strength 0.18), the ionic interaction of ribonuclease A (bovine), chymotrypsinogen A and cytochrome *c* with up to 100 μM polymer was nearly completely suppressed. Lysozyme still showed some interaction under these conditions. If an affinophore molecule bears 20 ligands, 100 μM of it would be sufficient to cause half of the maximum mobility change for a specific protein with the intrinsic dissociation constant of 2 m*M* for the ligand. For a high-affinity system, buffers of lower ionic strength can be used, and this is desirable for rapid completion of electrophoresis.

The ionic interaction can be positively utilized in "mobile ion-exchange electrophoresis". Succinylpolylysine is usable as a soluble ion exchanger for the separation of proteins. Haemoglobin and glycated haemoglobin were separated by using dextran sulphate as a soluble ion-exchanger[44].

3.3. Practical guide for the preparation of affinophores

At present, polylysine is the most convenient base polymer for affinophores, as it is commercially available as fractionated polymers differing in degree of polymerization, and the reactivity of its amino group is very useful for derivatization to the affinophore. Although polylysine with an average degree of polymerization of 120 or 190 has been used, the relationship between the size of the polymer and the effectiveness of an affinophore has not been studied. Large affinophores may be suitable for the affinophoresis of a protein of high molecular weight and small ones for affinophoresis in a small-pore gel such as polyacrylamide gel.

An affinophore can be either cationic or anionic in principle. However, as mentioned above, the use of cationic affinophores poses some practical problems in agarose gel electrophoresis. First, cationic affinophores are adsorbed on agarose, probably owing to an ionic interaction with sulphate or carboxyl groups on agarose, and second, they are stained with a dye such as Coomassie Blue and hence they interfere with the detection of proteins in the gel. When the ligand was benzamidine or tryptophan, even anionic affinophores with carboxyl groups were stained by Coomassie Blue dye. Although the factors determining the affinity of the dye have not been fully identified, the importance of cationic or aromatic functional groups was suggested by the results of an experiment using synthetic polyamino acids[45]. A change in the ionic group from carboxylate to sulphate made the affinophores no longer stainable with the dye. The tryptophan–affinophore in which succinyl groups were fully modified with aminomethanesulphonic acid slightly interfered with the staining of proteins by the anionic dye, probably because of ionic repulsion. Partial coupling of aminomethanesulphonic acid (about one fifth of succinyllysine residues or some excess over a cationic ligand) may thus be advisable.

The following procedure requires ligands that have an amino or a carboxyl group available for coupling, *i.e.*, these groups should not be a determinant of specificity (Fig. 8). The coupling reactions are mainly based on the formation of amide bonds by means of a water-soluble carbodiimide, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide[21]. The amount of the polymer can be determined by amino acid analysis

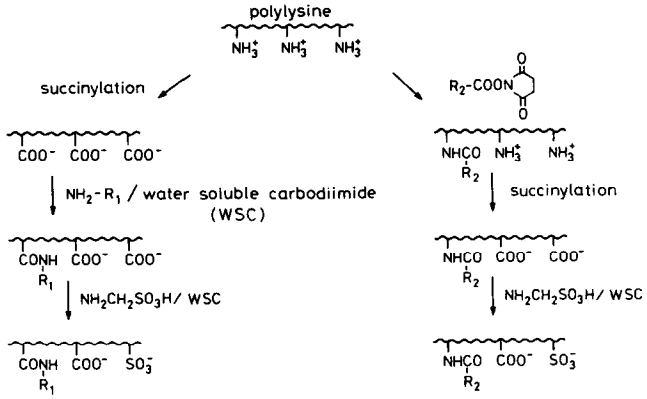

Fig. 8. Preparation of anionic affinophores based on polylysine. NH_2–R_1, an affinity ligand having an amino group available for coupling; R_2–COOH, an affinity ligand having a carboxyl group available for coupling.

after hydrolysis[32]. The coupling of amino ligands is carried out in an aqueous solution (pH 4.5–5) of succinylpolylysine and a ligand (10–20 mol-% of succinyllysine residue) by adding the carbodiimide (50 mol-% of succinyllysine residue). Aliphatic amines with high pK_a values are less reactive than arylamines, amino acid esters or aminomethanesulphonic acid under these conditions. In this instance, the use of 0.2 *M* morpholinopropanesulphonic acid buffer (pH 7) is recommended. The coupling reaction should be monitored by following the disappearance of the free ligand. For a carboxyl ligand, direct coupling to polylysine by means of the carbodiimide did not afford satisfactory results, probably because of the high pK_a value of the ε-amino group of the lysine residues. In this case, the carboxyl group of the ligand should be activated beforehand as the N-hydroxysuccinimide ester. After the coupling of the activated ligand to polylysine in 0.1 *M* sodium phosphate buffer (pH 7.5), the polymer is succinylated. Aminomethanesulphonic acid is coupled by means of water-soluble carbodiimide as for amino ligands at pH 4.5–5.

Polylysine and succinylpolylysine are both highly soluble in water. The reaction can be carried out at high concentration (20 mg/ml) and thus is completed very rapidly with high coupling yields. The carbodiimide can be used in excess over the ligand, as most of the excessively activated carboxyl groups are hydrolysed back to carboxyl groups[21]. After the reactions, polymers can be readily purified by dialysis. The affinophores prepared based on succinyl polylysine had mobilities of 1.0–1.3 relative to bromophenol blue at pH 8.

3.4. Two-dimensional affinophoresis

One-dimensional affinophoresis gives satisfactory results for samples of simple composition. For complex samples, two-dimensional affinophoresis makes the identification of a specific protein very easy. The first electrophoresis is carried out without an affinophore and the second is carried out at right-angles to the first under identical conditions except for the presence of an affinophore. Non-specific proteins should lie on a diagonal line and the specific protein should be found away from the line, as its mobility is changed in the second electrophoresis by the affinophore. Even if the change in mobility is not large, the deviation can be readily observed.

An extract of pancreatin, a dry preparation of porcine pancreatic juice, was subjected to two-dimensional affinophoresis with the anionic benzamidine–affinophore based on polyacrylyl-β-alanyl-β-alanine[46]. After the first electrophoresis in a slab of 1% agarose gel, the gel containing the affinophore was formed on the side of the separated proteins and the second electrophoresis was carried out so that the affinophore would migrate over the protein samples (Fig. 9). Such a two-dimensional affinophoresis can be completed within 1.5–2 h. Coomassie Blue staining revealed that a doublet spot was separated from the diagonal line (Fig. 10A). Prior to protein staining, a sheet of filter-paper impregnated with a fluorogenic substrate for trypsin was placed on the gel for a short period in order to absorb a part of the solution in the gel. Incubation of the paper developed a double fluorescent spot at the position corresponding to the separated spots revealed by protein staining (Fig. 10B). When the matrix polyionic polymer polyacrylyl-β-alanyl-β-alanylaminomethanesulphonic acid was used instead of the affinophore at an equivalent concentration, deviation of trypsin from the diagonal line was not observed. *S. griseus* trypsin in pronase was also separated by two-dimensional affinophoresis[46].

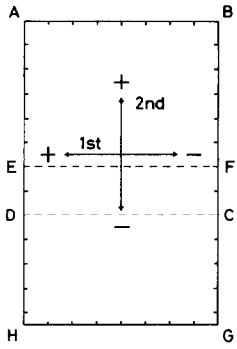

Fig. 9. Construction of gels for two-dimensional affinophoresis. The first-dimensional electrophoresis was carried out in a square gel without an affinophore (the square ABCD, 80 × 80 mm). A sample solution was applied in a hole at 25 mm from edge CD. Prior to the second-dimensional electrophoresis, part of the gel (the rectangle CDEF) was cut away and the gel containing an affinophore was formed (the rectangle EFGH). The gels were 1 mm thick. The edges of the diagram are notched at 10 mm intervals. (From ref. 46.)

The extracts of legume seeds were subjected to two-dimensional affinophoresis with an anionic affinophore bearing α-D-mannoside as an affinity ligand in order to separate mannose-binding lectins[47]. The affinophore was prepared by coupling *p*-aminophenyl-α-D-mannoside (10 mol-% of lysine residue) and aminomethane-

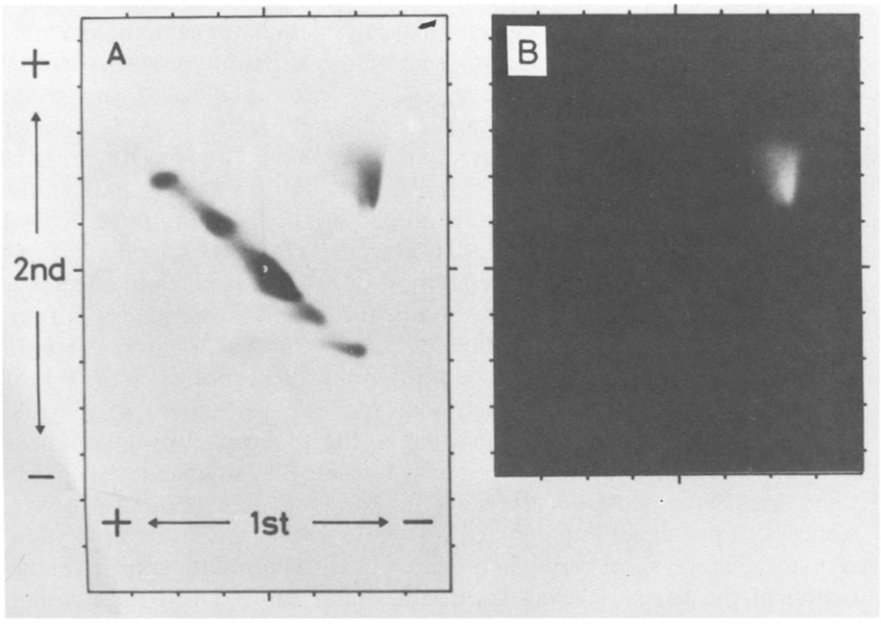

Fig. 10. Separation of porcine trypsin from pancreatin by two-dimensional affinophoresis. The extract (2 μl) of pancreatin was applied in a hole 5.5 cm from the top, 4 cm from the sides. Electrophoresis was carried out for 20 min at 60 mA per plate (about 25 V cm^{-1}) in each direction by using 0.1 *M* Tris–acetic acid buffer (pH 7.85). The concentration of the affinophore was 0.02%. (A) Coomassie Brilliant Blue R250 staining; (B) detection of trypsin activity with a fluorogenic substrate. (From ref. 46.)

sulphonic acid (20 mol-% of lysine residue) to succinylpolylysine (degree of polymerization 120).

Pea seed contains a lectin specific for D-mannose or D-glucose[48]. The lectin has a molecular weight of about 50 000 and has two sugar-binding sites. Two-dimensional affinophoresis of the extract of pea seed separated a spot from the diagonal line (Fig. 11A). Immuno-staining of the protein blotted onto a nitrocellulose membrane after the affinophoresis with anti-pea lectin antibody showed that the spot was the lectin (Fig. 11B). Blotting of separated proteins from the agarose gel is very easily performed and 10 ng of the lectin could be detected. In the presence of a free ligand, methyl-α-D-mannoside, the spot did not migrate away from the diagonal line (Fig. 11C and D). This shows the specificity of the affinophoresis. A lectin was also separated from the extract of fava bean in the same way. In the case of jack bean, a similar experiment resulted in the formation of a dense precipitate in the second electrophoresis. The problem was resolved by decreasing the amount of the sample to one-tenth. The abundance of the lectin, concanavalin A, in the bean might create conditions favourable for the formation of the specific aggregate of the affinophore and the lectin.

Anti-hapten antibody in rabbit serum was separated by two-dimensional affinophoresis directly from antiserum[49]. The hapten was a tripeptide with a blocked amino terminus, N-(dibenzyloxyphosphinoyl)-L-alanyl-L-prolyl-L-proline. The affinophore was prepared by reaction of the N-hydroxysuccinimide ester of the N-blocked tripeptide with polylysine having an average degree of polymerization of 190. The polymer was then succinylated and the coupling was carried out with aminomethanesulphonic acid. The content of the ligand was 15 mol-% of lysine residue and that of aminomethanesulphonic acid was about 20 mol-%. The specific anti-hapten antibody separated by the affinophoresis was detected by Coomassie Blue staining and immunostaining.

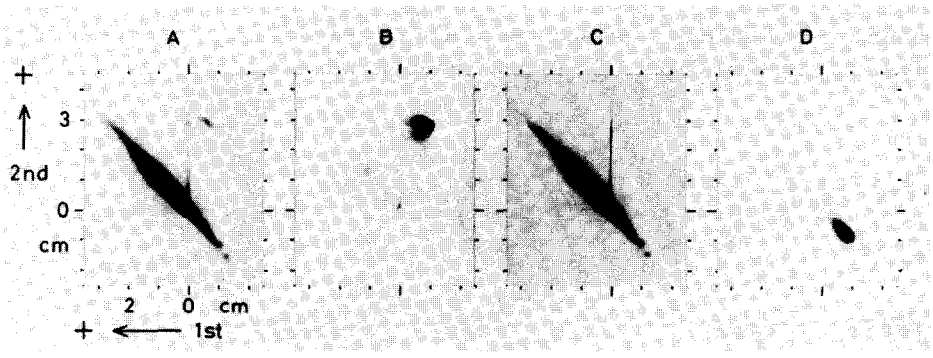

Fig. 11. Two-dimensional affinophoresis of the extract of pea seed with the mannose–affinophore. The sample was applied at the position 0 and electrophoresis was carried out for 30 min at 25 V cm^{-1} (40–50 mA per plate) in each direction by using 0.1 *M* Tris–acetic acid buffer (pH 7.9) in (A and B) the absence or (C and D) the presence of 0.1 *M* methyl-α-D-mannoside. The concentration of the affinophore was 5.2 μ*M* (58 μ*M* for the ligand). A and C, 2 μl of the extract were applied and stained with Coomassie Brilliant Blue R250; B and D, 2 μl of the 10-fold diluted extract were applied and immunostaining was carried out after blotting onto a nitrocellulose membrane. Only the central part of gels containing proteins is shown. (From ref. 47.)

4. AFFINOPHORESIS OF CELLS

The difference in the electrophoretic mobilities of different cells is not always large enough to allow a clear separation by the usual electrophoresis. The alteration of the mobility of specific cells by affinophoresis might therefore be useful for the separation of specific cells by electrophoresis. Each cell population expresses distinct surface antigens and thus affinophoresis specific for the surface antigens should be effective.

4.1. Attachment of affinophore to surface antigens

For the preparation of an affinophore, the direct chemical conjugation of an antibody to an ionic polymer is a straightforward approach in principle. A preliminary attempt showed that direct coupling by using water-soluble carbodiimide resulted in a considerable loss of the binding ability of proteins, probably because of the formation of multiple bonds. As an alternative, an indirect method was emoloyed[50]. The procedure consists in four steps of treatment of cells with an antiserum, biotinylated second antibody, avidin and biotinylated succinylpolylysine as an affinophore (Fig. 12). The biotin–affinophore was prepared by reaction of the N-hydroxysuccinimide ester of *d*-biotin with polylysine, followed by succinylation. The anionic affinophore was used in order to avoid the expected non-specific ionic adsorption on the negatively charged cell surface. The procedure is readily applicable to different types of cells simply by changing the antiserum. The affinophoresis of cells was carried out at a non-equilibrium state of binding, unlike the case with proteins, owing to the low dissociation rate of the antigen–antibody system and avidin–biotin system.

4.2. Affinophoresis of red blood cells

Affinophoresis of red blood cells (RBCs) from rabbits, humans and rats was carried out and the electrophoretic mobility of cells was determined by using an automated cell electrophoresis analyser, CEP-1 (Shimadzu, Kyoto, Japan)[51]. Electrophoresis of all of the RBCs was accelerated when a homologous antiserum was used (Fig. 13). The largest effect, a 2.9-fold acceleration, was observed for rabbit RBCs,

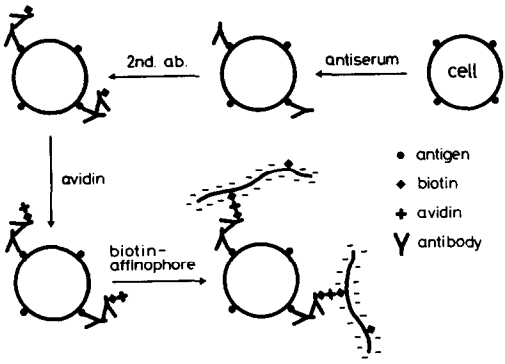

Fig. 12. Coupling of biotin–affinophore to cell. (From ref. 50.)

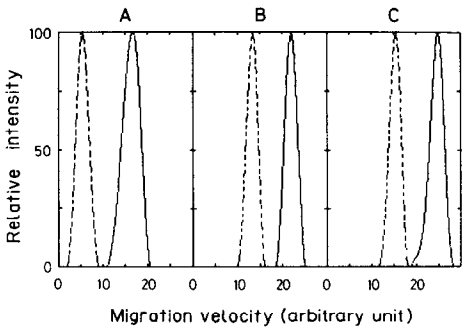

Fig. 13. Affinophoresis of red blood cells. The results of the electrophoresis of non-treated cells (broken lines) and those of affinophoresis (solid lines) are superimposed for each species. Electrophoresis was carried out with an automated cell electrophoresis analyser in 10 m*M* sodium phosphate buffer (pH 7.2) containing 0.13 *M* NaCl and 5 m*M* KCl at 25°C. The effect of electroosmotic flow is corrected. A, Rabbit; B, human; C, rat. (From ref. 50.)

which have the lowest original mobility among the three. The effect of affinophoresis depended wholly on the specificity of the antiserum used, since no acceleration was observed when a non-immune serum was used instead of the homologous antiserum. The affinophoresis increased the mobility of RBCs of human and rat 1.7- and 1.6-fold, respectively. The largest amount of antiserum should be used for the largest effect, provided that cell aggregates are not formed. The other three reagents, biotinylated second antibody, avidin and biotin–affinophore, can be used in amounts that give the plateau levels of the dose–response relationship.

A larger affinophore, with an average degree of polymerization (DP) of 1150, was more effective than a smaller one (DP of 270). For an affinophore of a given size, the lower the biotin content the more effective it was. The most effective affinophore had an average of seven biotinyl residues on one succinylpolylysine molecule with an average DP of 1150.

To test the feasibility of affinophoresis for specific cell separations, two types of RBCs were mixed and subjected to affinophoresis by using either of the two corresponding antisera. Electrophoresis of an untreated mixture of rabbit and human RBCs gave two peaks (Fig. 14, A1). The peaks were assigned by comparing the data with those obtained for each single species presented in Fig. 13: the peak of lower velocity for the rabbit and that of higher velocity for humans. Affinophoresis of the same mixture after treatment with anti-human RBC serum resulted in specific acceleration of human RBCs and thus the distance between the two peaks was extended (Fig. 14, A3). The use of anti-rabbit RBC serum gave a single peak, probably because of the specific acceleration of rabbit RBCs and the consequent overlapping with human RBCs (Fig. 14, A2). Similar results were obtained for the combination of rabbit and rat RBCs (Fig. 14, C). The mixture of non-treated human and rat RBCs gave a single peak because of the small difference in the original migration velocities of the two (Fig. 14, B1). By using either antiserum, the affinophoresis gave two peaks (Fig. 14, B2, B3). The peak which has the higher velocity in each histogram must be that of the RBCs homologous with the antiserum used. The clearer separation of the peaks attained with the anti-rat RBC serum accords with this interpretation, as rat

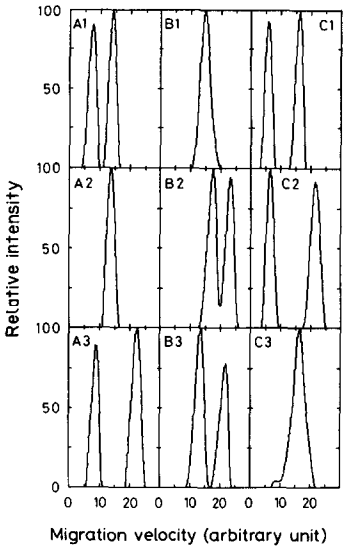

Fig. 14. Affinophoresis of mixed red blood cells. RBCs from two spcies were mixed and electrophoresis was carried out before or after treatment for affinophoresis. Other conditions were the same as in Fig. 13. A, Rabbit and human; A1, no treatment; A2, affinophoresis with an anti-rabbit RBC serum; A3, affinophoresis with an anti-human RBC serum. B, Human and rat; B1, no treatment; B2, affinophoresis with an anti-human RBC serum; B3, affinophoresis with an anti-rat RBC serum. C, Rat and rabbit; C1, no treatment; C2, affinophoresis with an anti-rat RBC serum; C3, affinophoresis with an anti-rabbit RBC serum. (From ref. 50.)

RBCs originally showed a higher velocity than human RBCs. The last results show the great potential value of affinophoresis in specific cell separations, which cannot be achieved by ordinary electrophoresis.

5. PROSPECTS

For the affinophoresis of proteins, insoluble supports for electrophoresis other than agarose gel may offer specific advantages. The use of a cellulose acetate membrane would further simplify the procedure. If an affinophore is reasonably small, the use of polyacrylamide gel and discontinuous buffer systems[52] could afford a higher resolution than the agarose gel system. Affinophoresis would make a good combination with capillary electrophoresis.

For a preparative application of affinophoresis, two-step electrophoresis would be effective. This is identical with two-dimensional affinophoresis in principle. First electrophoresis is carried out without the affinophore and then the fraction containing the target substance is subjected to a second electrophoresis under identical conditions except for the presence of the affinophore. The target substance would be transported at the anodic side of the other substance if an anionic affinophore is used. A discontinuous buffer system, *e.g.*, chloride ion as the leading ion and glycine as the trailing ion, may be useful in some instances. If experimental conditions are chosen in order that an affinophore–protein complex is transported at the interface of ions, it would be possible to retrieve the target substance in a highly concentrated state.

At present, a preparative electrophoresis apparatus that is usable for cell separation is still costly and its operation is not straightforward. As electrophoresis is simple in itself, the development of apparatus that is simpler and easier to operate than the present version is likely. In that event affinophoresis should become a method of choice for separating specific cells, in addition to ASECS.

Affinophoresis is a versatile method and the results described here are only a starting point. The scope for future developments is enormous.

6. ACKNOWLEDGEMENT

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7. ABSTRACT

The use of polyionic polymers as mobile affinity matrices in electrophoresis has led to the development of a specific separation method for biological substances, affinophoresis. The conjugate of a polyionic polymer and an affinity ligand is called an affinophore. Electrophoresis of proteins in the presence of an affinophore results in a change in the mobility of a specific protein due to the difference between the mobility of the protein and that of the protein–affinophore complex. Polylysine is useful as a base polymer of affinophores and has been used successfully as an anionic matrix after succinylation. Affinophoresis of proteases, lectins and antibodies has been carried out in agarose gel and the mobility of the protein having affinity to each ligand was specifically changed. Two-dimensional affinophoresis, in which an affinophore was included only in the second-dimensional electrophoresis, was effective for the separation of the components of a complex mixture of proteins even if the change of mobility was not large. Red blood cells were successively treated with homologous antiserum, biotinylated second antibody, avidin and biotinylated succinylpolylysine as an affinophore. Specific acceleration of the homologous cells to the antiserum was observed even when the affinophoresis was applied to mixed red blood cells from different species.

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