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PREPARATIVE PLASMA PROTEIN FRACTIONATION BY ISOTACHOPHORESIS IN SEPHADEX COLUMNS

M. BIER, R. M. CUDDEBACK and A. KOPWILLEM*

Veterans Administration Hospital and University of Arizona, Tucson, Ariz. 85723 (U.S.A.)
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SUMMARY

The use of Sephadex G-200 as a stabilizing medium for preparative isotachophoresis (ITP) has been investigated and the resolution was compared to that previously obtained in the customarily used polyacrylamide gels. Human plasma proteins were fractionated in an LKB Uniphor column, using a variety of amino acids and peptides as discrete spacers, which separate the sample into clearly defined protein zones. Immunoelectrophoretic analysis of ten specific plasma components has shown that the majority of proteins were sharply resolved, but there was some trailing or overlap for other proteins. The spacing characteristics of amino acids and peptides were found to be dependent on the pH of the leading electrolyte, thus adding to the flexibility of preparative ITP for the isolation of protein subgroups with a desired mobility range. The resolution on Sephadex was comparable to that on polyacrylamide, though the mobility of spacers was different, as was to be expected. Sephadex columns are, however, easier to prepare, more reproducible, and do not exhibit any of the boundary anomalies frequently present in ITP on polyacrylamide.

Analytical ITP in free solution in the LKB Tachophor proved to be of value for the identification and quantitation of the amino acid spacers in the preparative fractions. It also permitted the determination of the relative mobilities of the separated proteins. On the basis of the immunoelectrophoretic data, it was possible to assign specific proteins to some of the bands visible in the complex Tachophor recordings.

INTRODUCTION

In a recent paper in this journal we have demonstrated the usefulness of amino acids and simple peptides as discrete spacers in preparative isotachophoretic fractionation of serum proteins[1]. This was an extension of the earlier finding of Vestermark[2] that amino acids have net mobilities comparable to those of proteins in analytical isotachophoresis (ITP). ITP is characterized by the use of a discontinuous buffer system, where the sample is separated into sharply defined compartments

* Present address: LKB-Produkter AB, Bromma, Sweden.

formed between a high-mobility leading electrolyte and a low-mobility terminating electrolyte[3–14]. These sample compartments remain contiguous to each other, unless separated by spacer ions of intermediate net mobility. Ampholines are customarily used as spacers, but they provide a nearly continuous spectrum of mobilities[15], thus causing an indiscriminate stretching of the ITP protein patterns. Amino acids, simple peptide or other discrete spacers of appropriate mobilities provide an appealing alternative, as they cause selective spacing of the protein patterns[1,2].

Because of the large density gradients created by the sharp sample boundaries, stabilizing media are needed and polyacrylamide gels have been routinely used for preparative work[8–14]. Unfortunately, unpredictable distortions of boundaries are frequently seen and they seem to aggravate as protein loading is increased[1]. A quest for alternate stabilizing media seemed therefore desirable, and preliminary studies in this laboratory showed that good separation of colored proteins was obtainable using Sephadex for stabilization. While Sephadex G-10 and G-25 were found to be unsuitable, G-50 to G-200 seemed to be comparable, and G-200 was arbitrarily chosen for this study.

Sephadex had not been previously used for support in preparative ITP as it was probably anticipated that problems may arise due to its sieving characteristics. The purpose of the present paper is to demonstrate that, to the contrary, Sephadex appears to present many advantages over polyacrylamide gels. The main advantages are in the ease and reproducibility of column preparation, and the complete absence of boundary anomalies. As a result of the latter, protein loading could be substantially increased. As expected, sieving does cause a relative retardation of the mobility of the low-molecular-weight spacers, thus, to obtain comparable spacings, faster amino acids have to be used on Sephadex. But otherwise the results are comparable, and, as on polyacrylamide, on Sephadex also n spacers sharply fractionate the majority of the serum proteins into $n + 1$ fractions, *i.e.*, mobility subgroups. Some proteins remain polydisperse, as was previously observed for polyacrylamide, and, in this respect, Sephadex proved to be of no advantage.

In the present paper data are presented on the spacing characteristics of various amino acids and dipeptides on Sephadex as a function of the pH of the leading electrolyte. The data are compared with those obtained in free solutions, using analytical ITP. Analytical ITP proved to be also advantageous for the analysis of collected fractions and their amino acid content.

MATERIALS AND METHODS

Reagents

Cacodylic acid, tris(hydroxymethyl) aminomethane (TRIS), $Ba(OH)_2$, and sucrose were obtained from Fisher Scientific (Pittsburgh, Pa., U.S.A.); Sephadex G-200 from Pharmacia (Uppsala, Sweden); hydroxypropylmethylcellulose, of 15 000 cps viscosity (Methocel K 15M Premium) from Dow Chem. (Midland, Mich., U.S.A.); amino acids from Sigma (St. Louis, Mo., U.S.A.); glycylglycine and N,N-bis-(2-hydroxyethyl)-glycine (bicine) from BDH (Poole, Great Britain); agarose (Indubiose A45) from L'Industrie Biologique Francaise (Gennevilliers, France); and human antisera (rabbit) from Behring Diagnostics (Somerville, N.J., U.S.A.).

Sample

Outdated plasma from the Red Cross was used to prepare an albumin poor protein fraction. This fraction was prepared by precipitation at 50% saturation of ammonium sulfate. The precipitate was dissolved in water and dialyzed against the leader buffer. Its protein concentration was 1.8% (experiments 2–4) or 2.3% (experiments 1 and 5).

Preparative ITP in Sephadex

The separations were performed in the LKB Uniphor apparatus, with Sephadex G-200 as the stabilizing medium. The 40 cm long column had an I.D. of 3.5 cm and was kept at 12°. Sephadex was equilibrated in the leading electrolyte and poured to an average column height of 20 cm. The spacing properties of six amino acids and peptides, *i.e.*, asparagine, bicine, glutamine, glycine, glycylglycine, and threonine, were investigated, these spacers covering the mobility range of plasma proteins. The leading electrolyte was 20 m*M* cacodylic acid, adjusted to pH 7.1 (experiments 2, 3, and 6), or pH 8.1 (experiments 1, 4, and 5) with TRIS. Density stabilization of the leader was brought about by the addition of 10% sucrose. The terminator was in all experiments 100 m*M* β-alanine, brought to pH 9.2 with $Ba(OH)_2$. $Ba(OH)_2$ was added to precipitate bicarbonate ions, and previous experience with analytical ITP had shown that the addition of TRIS counterion is not necessary in the terminator.

Upon application of the sample, the initial voltage was set to 600 V (approximately 20 mA) to allow migration of the sample into the gel, and was increased to 1200 V after 4 h. The current decreased progressively to about 5 mA at 1200 V towards the end of the run. The time for separation was typically 20 h (4 at 600 V and 16 at 1200 V), until the first protein boundary was eluted. Complete elution required about 7–15 h, depending on the zone lengths in the column, *i.e.*, quantity of protein and spacers applied.

The leading electrolyte was used as elution buffer at a rate of 13–17 ml/h, depending on the experiment. The eluent was monitored using the LKB 8300 Uvicord at 280 nm, and fractions were collected at 10 min (experiment 6) or 15 min (experiments 2–5) intervals. The sample dilution due to elution was approximately 1:3.

Analytical ITP in free solution

The LKB 2127 Tachophor was used to analyze the fractions collected from the Uniphor. The instrument operates at constant current and is equipped with a UV and a thermal detector. The separations were performed in a 24 cm × 0.5 mm I.D. PTFE capillary, in free solution, using 0.5% hydroxypropylmethylcellulose as stabilizer. The UV detector recorded the migration of the protein zones, while the thermal detector recorded also non-UV absorbing zones, the temperature in each zone being a function of the net mobility of the ionic species in that zone, due to the stepwise increase in voltage gradient characteristic of ITP. The apparatus requires more dilute solutions for boundary stability than the Uniphor column, and the leading electrolyte was 5 m*M* cacodylic acid, adjusted to pH 7.1 or 8.1 with TRIS. The terminating electrolyte was in all cases 10 m*M* β-alanine with $Ba(OH)_2$ added to pH 9.2. The samples were injected at the boundary between the leading and the terminating electrolyte, together with the same six individual spacers as used in the preparative

experiments. The capillary was thermostated at 20° and a constant current at 60 μA was applied. The voltage increased from 2 to 22 kV with the leading electrolyte of pH 7.1, and from 2 to 15 kV with the leading electrolyte of pH 8.1.

Immunoelectrophoretic analysis

The protein fractions collected from the Uniphor were analyzed for ten specific proteins. A modified Laurell[16] rocket technique was used to analyze nine of them. Precoated glass slides, 8 cm × 10 cm, were layered with 10 ml of 0.85% agarose in a 5 m*M* HCl–TRIS buffer (pH 7.4). Only 0.1 ml of monospecific antisera/10 ml of agarose was used, corresponding to a tenfold decrease of antisera as compared with the original method. IgG was analyzed by immunodiffusion as outlined by Mancini *et al.*[17] using the same buffer as above except that the antiserum concentration was 0.25 ml/10 ml. Sample quantities of 2 μl were applied in both techniques. Calibration curves were established by analyzing dilutions of the original plasma protein fraction and the data are reported in Figs. 2–6 as percentage of the original.

RESULTS

A typical photograph of an isotachophoretic separation in the Uniphor apparatus of our plasma protein preparation is shown in Fig. 1. Sephadex G-200 was used as the supporting medium, and the leading electrolyte was 20 m*M* cacodylic acid, pH 8.1. Asparagine, threonine, glutamine, and glycine were used as discrete mobility spacers, having intermediate net mobilities between those of the leading cacodylic acid and the terminating β-alanine. It is clearly evident that these spacers are effective in separating the proteins into sharply defined zones. The first zone, between cacodylic acid and asparagine, was intensely yellow, due to albumin, and was about 2 mm in length. The second, 4 mm long zone, was red, colored by traces of hemoglobin present in the starting sample. This was followed by a broader, faintly reddish zone containing the spacer threonine; thus, with this spacer complete separation of protein and spacer was not achieved. The last two protein zones were mainly visible due to their intense refractive index gradients. The results confirmed the expectation that four discrete spacers will separate a complex protein mixture into five zones. Zones 1, 4, and 5 were extremely sharp, zone 2 less so, while there was no observed zone between threonine and glutamine, the third protein zone being mixed with threonine. Better resolution of this zone may be obtainable on longer separation time. All spacer zones were much longer than the protein zones, thus facilitating elution.

This photograph is illustrative of all separations on Sephadex G-200, inasmuch as the protein zones are always flat and do not exhibit the anomalies frequently observed on polyacrylamide gels[1]. Sephadex gels being only semitransparent, visual inspection may not be sufficient to ascertain if there is uniform protein distribution within the planes of each protein zone, but no irregularities were found on extrusion of a number of column contents.

For the characterization of specific protein distribution in the collected fractions, simpler systems were chosen, using spacers with greater differences in their net mobilities. Two such experiments are reported in Figs. 2 and 3, using glycylglycine and threonine in the first case, and bicine, asparagine, and glutamine in the second.

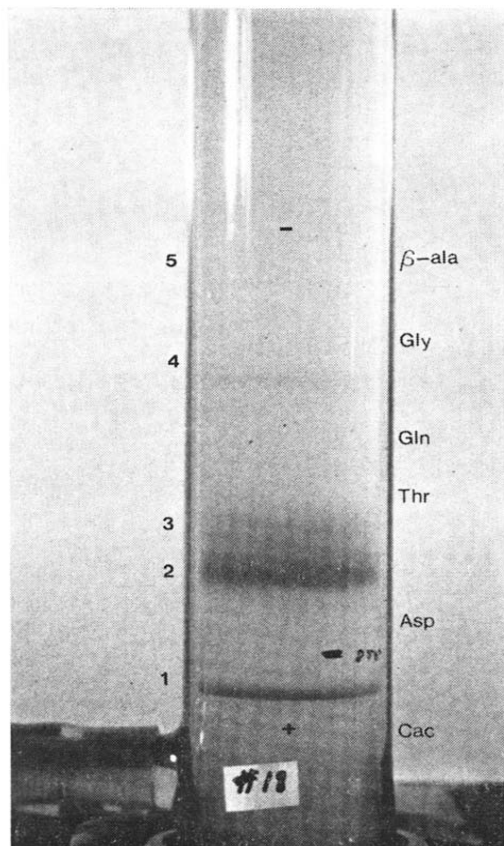

Fig. 1. Photograph of ITP on Sephadex of a sample containing 180 mg of the plasma protein preparation with 200 μmoles each of asparagine, threonine, and glutamine and 120 μmoles of glycine as discrete spacers. The starting sample volume was 28 ml. The leading electrolyte was 20 m*M* cacodylic acid, adjusted to pH 8.1 with TRIS; the terminator was 100 m*M* β-alanine. Zone 1 was yellow, zone 2 red, and zone 3 reddish, while the remaining two zones were mainly visible due to their intense index of refraction gradient. The spacer zones were about 2 cm long, while the protein zones had sharp boundaries and were only 2–4 mm long.

In both instances, the leading electrolyte was 20 m*M* cacodylic acid, pH 7.1, and β-alanine was the terminator, TRIS being the common counterion. The UV recordings of the eluates are presented in the top tracings, and the results of quantitative immunoanalysis in the bottom graphs.

Of the nine proteins analyzed in the experiment reported in Fig. 2, six were sharply separated by the spacers, albumin being confined to the first protein zone, the other five to the second zone. Of the three remaining proteins, α_1-antitrypsin migrated mainly with albumin, but was also found in the second protein zone, while the distribution of ceruloplasmin was reversed. The third protein, IgA, showed trailing throughout the threonine spacing zone. IgG analysis was omitted, but from other data it can be assumed that it constituted the main bulk of the third protein zone. The implications of this lack of sharp resolution will be discussed later on.

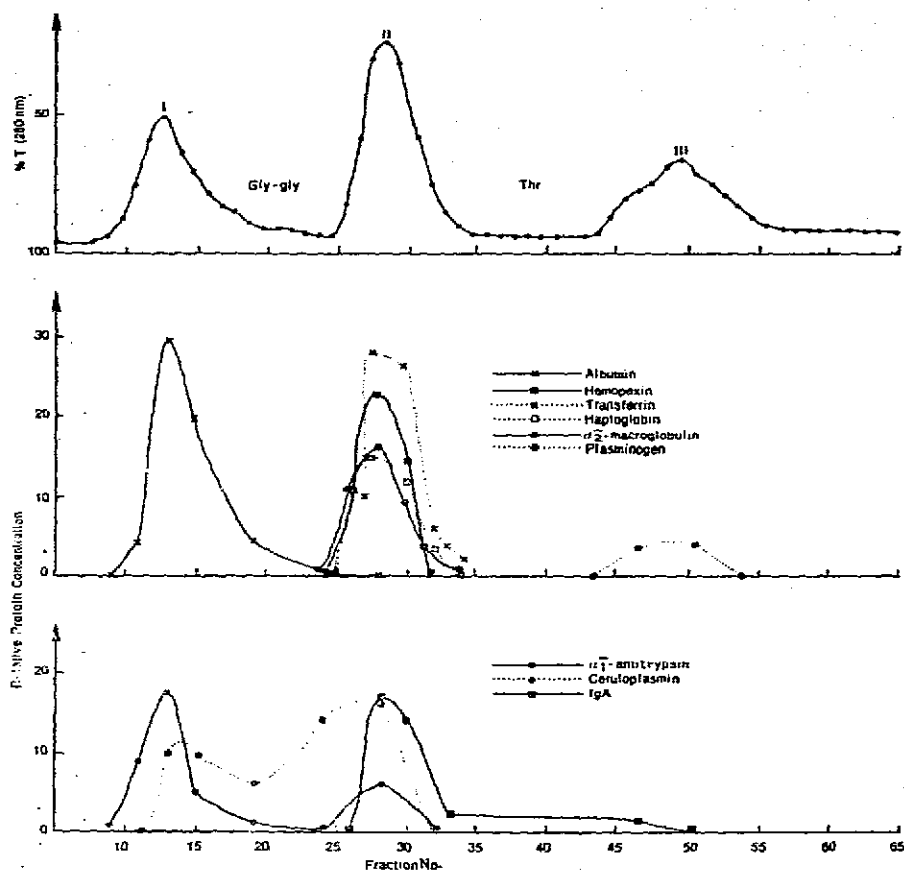

Fig. 2. Isotachophoretic separation of 230 mg of the plasma protein preparation with 140 μmoles of glycylglycine and 160 μmoles of threonine as spacers. The leading electrolyte was 20 m*M* cacodylic acid adjusted to pH 7.1 with TRIS; the terminator was β-alanine. The top graph reproduces the UV absorption of the column eluate by the Uvicord apparatus, while the two bottom graphs show the results of immunoelectrophoretic analysis of selected fractions. The data are reported as relative protein concentration in the individual fractions, with reference to the original plasma protein preparation.

In the second experiment reported in Fig. 3, the separation of proteins was rather comparable. Bicine, the first spacer, is somewhat slower than glycylglycine, and separated more sharply α_1-antitrypsin and ceruloplasmin. The distribution of α_2-macroglobulin can also be explained on the same basis. Asparagine has a higher mobility than threonine, and, as a result, the IgA component was clearly separated into two fractions. Finally, glutamine separated plasminogen and IgG from all other proteins, these being the only components found in the last zone.

With most proteins, the frontal boundary appears to be sharper than the rear boundary, which may be due to trailing in the elution system of the Uniphor. Trailing caused by elution may also explain the obvious discrepancy between the ratio of the lengths of protein to spacer zones visually seen in the columns (*cf.* Fig. 1)

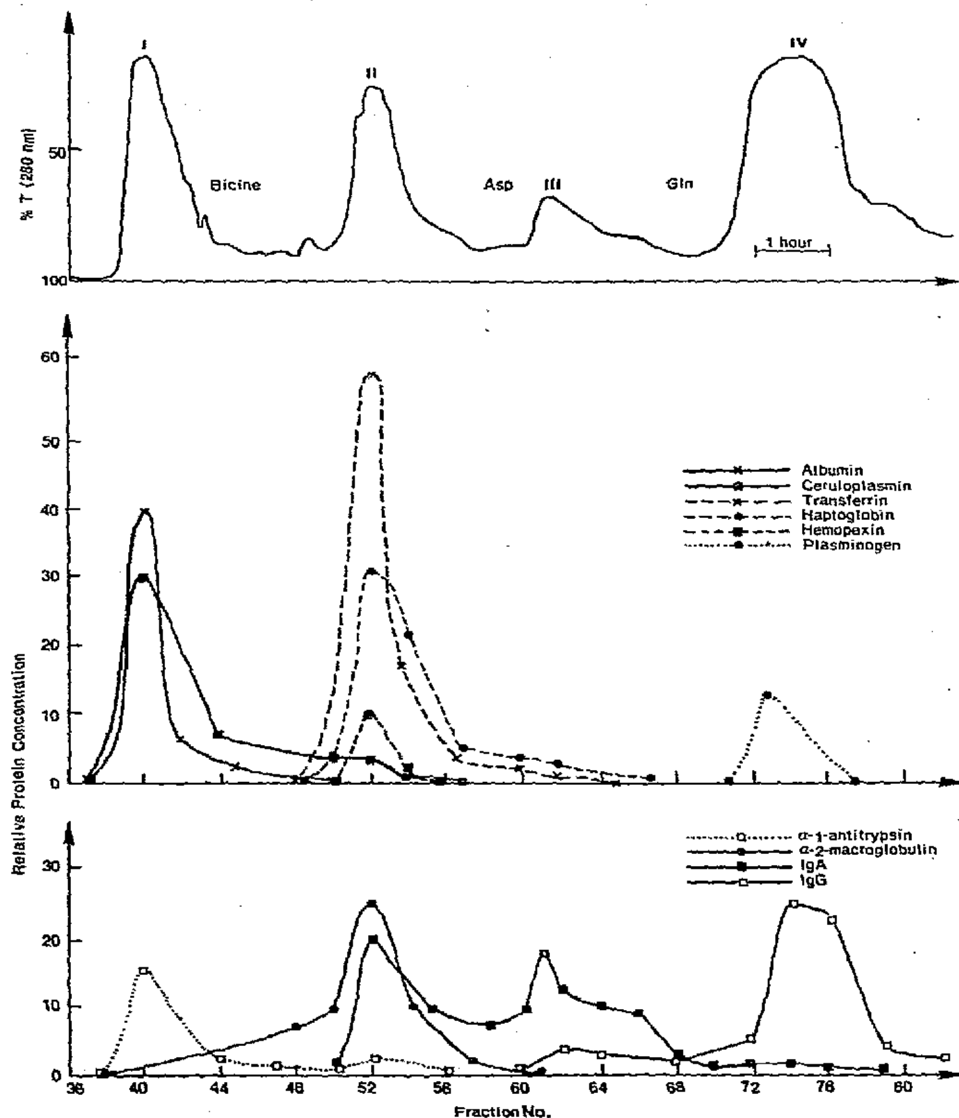

Fig. 3. Isotachophoretic separation of 180 mg of the plasma protein preparation using 240 μmoles of bicine, asparagine, and glutamine as spacers. Leader and terminator were as in Fig. 2.

and the UV tracings. On the columns the spacer zones are much longer than the protein zones, while this ratio is inverted in the UV tracings.

A change of the leading electrolyte from pH 7.1 to 8.1 radically changes the characteristics of the system, as it increases the net mobility of the spacers more than the mobility of the proteins. This is shown in Figs. 4 and 5, using the same spacers as in the previous two experiments. Both glycylglycine and bicine now have higher mobilities than any of the protein components, and migrate adjacent to the cacodylic acid. In Fig. 4, threonine was the first effective spacer, clearly separating in

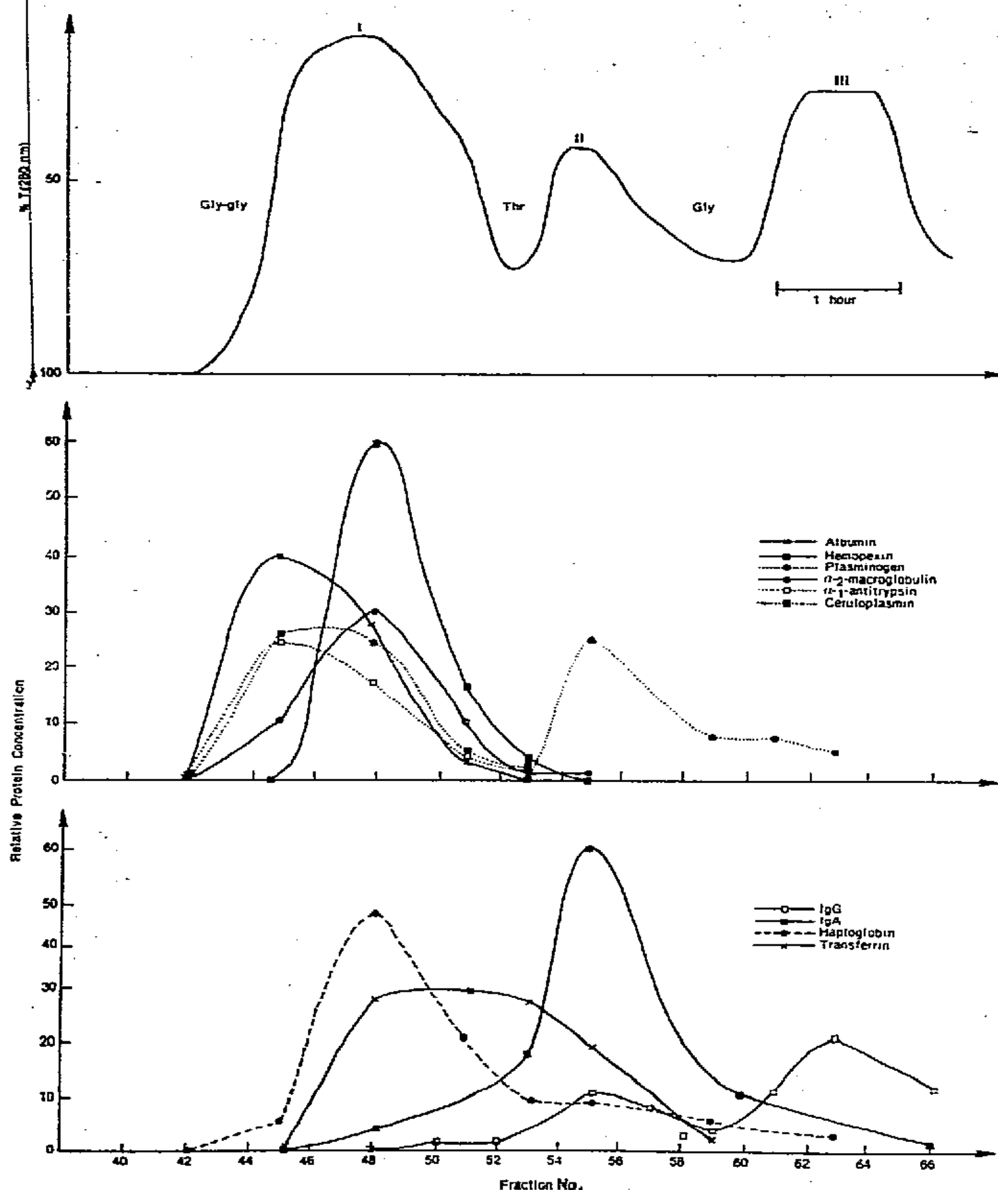

Fig. 4. Separation of 180 mg of the plasma protein preparation with 120 μmoles of glycylglycine and threonine and 80 μmoles of glycine as spacers. The leading electrolyte was 20 mM cacodylic acid, adjusted to pH 8.1 with TRIS; the terminator was the same as in the previous experiments. The UV tracing (top) of the eluate and the protein distribution (bottom) are shown.

the first zone five of the proteins examined. The two remaining proteins present in the first zone, haptoglobin and transferrin, showed considerable trailing into the second protein zone. This second zone also contained plasminogen and IgA, as well as a minor portion of IgG. The last zone contained mostly IgG, with traces of IgA and

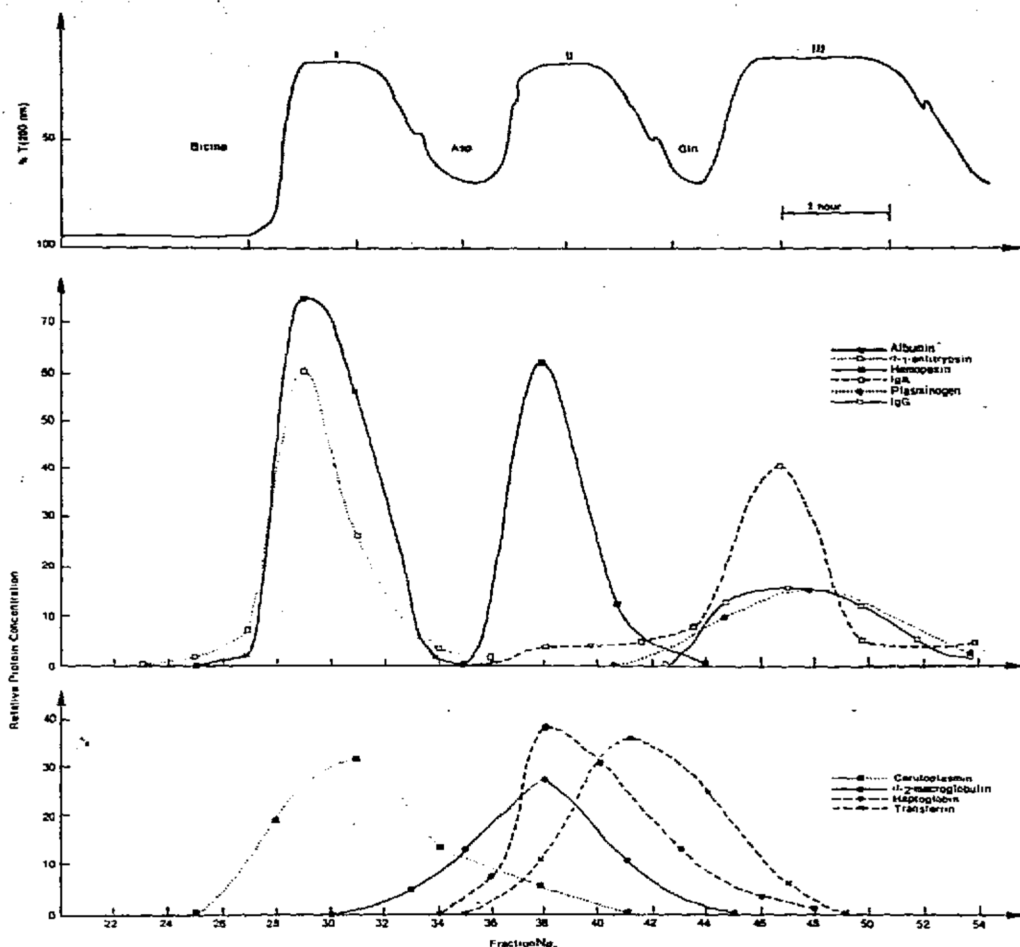

Fig. 5. Separation of 180 mg of the plasma protein fraction with 200 μmoles of bicine, asparagine, and glutamine as spacers. The other conditions and the presentation of results are the same as in Fig. 4.

plasminogen. The behavior of the remaining two spacers, asparagine and glutamine, at pH 8.1, is shown in Fig. 5. The six proteins shown in the center graph were well resolved, while there was substantial trailing for the four proteins shown in the bottom graph.

In all of the above experiments, the required number of protein bands was observed, in accordance with the number of effective amino acid spacers employed. Protein loading was mostly 180 mg per run, and the majority of the proteins assayed were well resolved, using two or three spacers. Less successful was an attempt to use simultaneously six spacers. For this run the protein load was increased to 280 mg and the results are shown in Fig. 6. At elution of the 20 cm long Sephadex column, the total length of the sample zones, between first and last boundary, was 9 cm. This was sufficient to resolve the spacers, but not the proteins. The UV tracing shows no signifi-

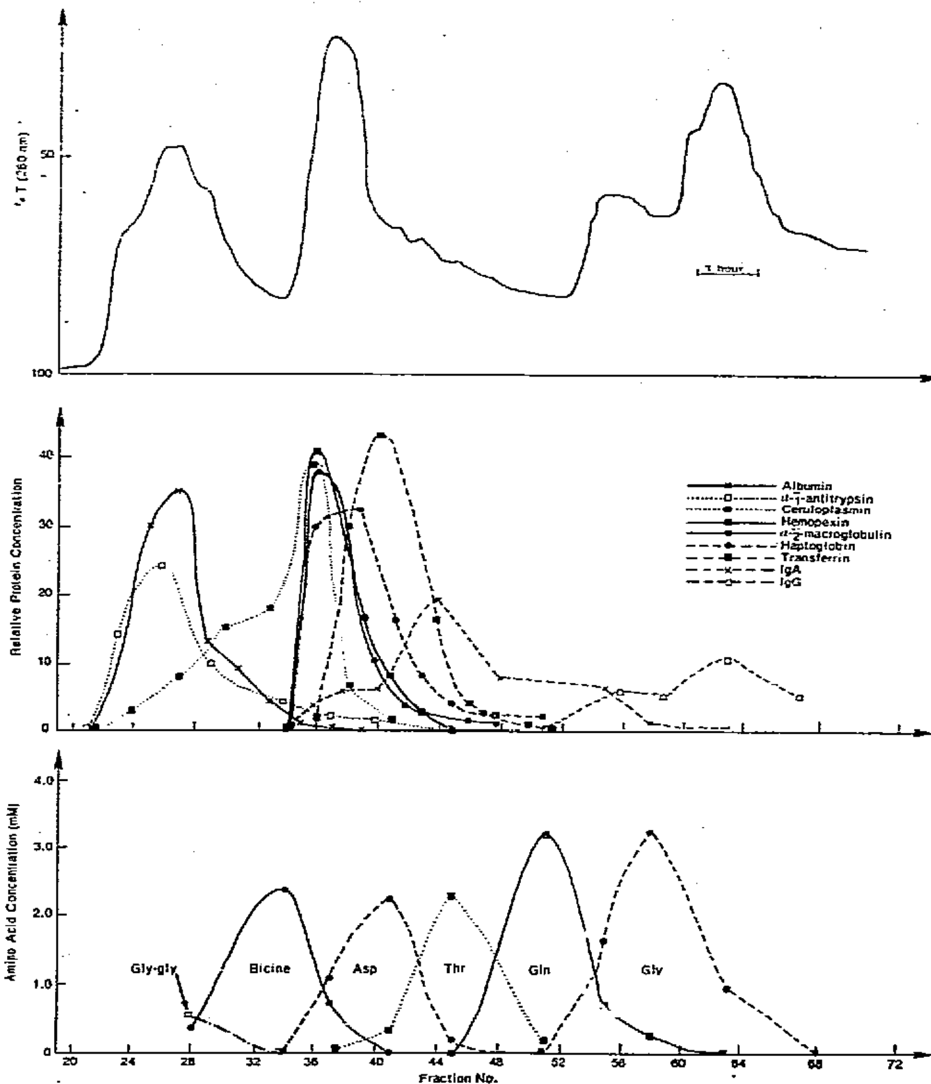

Fig. 6. Separation of 280 mg of the plasma protein preparation with 120 μmoles of glycylglycine, bicine, asparagine, threonine, glutamine, and 80 μmoles glycine. The leader pH was 7.1, the other conditions being as in earleir experiments. The top tracing shows the UV recording of the eluate, the center graph shows the protein distribution in selected individual fractions, and the bottom graph shows the amino acid distribution in the fractions.

cant improvement over that observed with three spacers only, and the immunoelectrophoretic analysis confirms the lack of good resolution. Most of the proteins remained grouped in the bicine to glutamine zones. A longer migration distance would probably have given better resolution.

Some of the Uniphor fractions were subjected to further isotachophoretic analysis in free solution, using the LKB Tachophor. As reference, a mixture of the six

amino acid spacers was utilized, and their Tachophor tracing is shown in Fig. 7A. The temperature tracing clearly identifies each amino acid, and the minor UV peaks are due to absorbing impurities unavoidable in most electrolyte systems. Fig. 7B shows the result of the addition of a plasma protein sample to this mixture of amino acids. Clearly, UV-absorbing components are now formed between all the non-UV absorbing spacer zones, the main bulk of the protein being located between the leading ion, cacodylate, and the fastest spacer, glycylglycine. In Fig. 7C the result is shown of the addition to the reference mixture of an aliquot of fraction 14, zone I, of the Uniphor experiment reported in Fig. 2. This fraction contained only albumin, α_1-antitrypsin and ceruloplasmin, and formed a zone between glycylglycine and cacodyl-

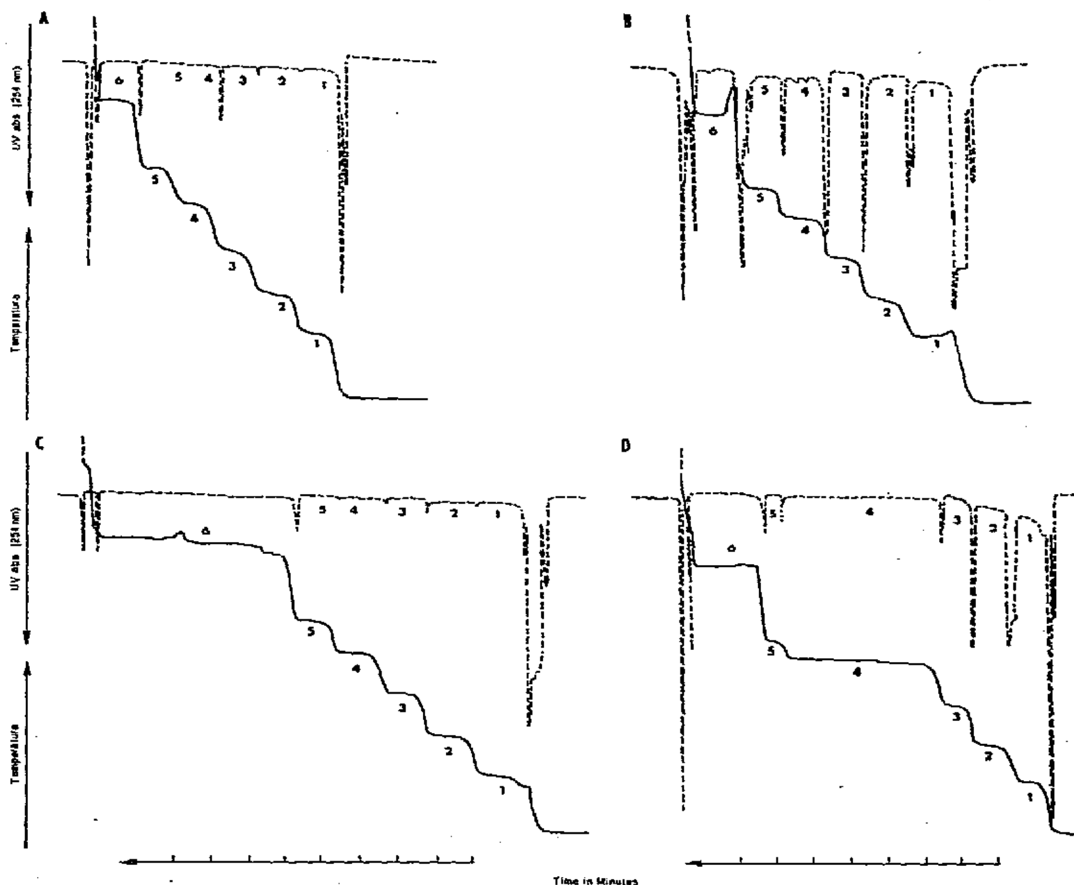

Fig. 7. Analysis with the Tachophor of the fractions obtained in experiment 2 (Fig. 2), in the presence of a reference mixture of spacers. (A) Spacer mixture alone of 2 μl each of glycylglycine (1), bicine (2), asparagine (3), threonine (4), glutamine (5), and glycine (6), all at 10 mM concentration. (B) 4 μl of the 2.3% original plasma protein preparation in the presence of the above spacer mixture. (C) 20 μl of fraction 14, zone I, in the presence of the above spacers. (D) 20 μl of fraction 30, zone II, and the spacer mixture. The leader was 5 mM cacodylic acid, adjusted to pH 7.1 with TRIS. The temperature and the UV absorption were recorded as the sample was migrating past the detectors. The leading ion is on the left of each plot, the terminator on the right. The analysis time varied from 30–60 min, depending on the salt concentration in the samples.

ate in the Tachophor, as in the preparative run. Step 6 of the temperature tracing is prolonged, probably due to slow impurities present in Sephadex. Fig. 7D shows the pattern obtained upon addition to the spacer mixture of an aliquot from fraction 30, zone II, of the same Uniphor experiment. It contained hemopexin, transferrin, haptoglobin, α_2-macroglobulin, ceruloplasmin, and IgA, and has been resolved in analytical ITP in three peaks, the main peak being between glycylglycine and bicine, thus being somewhat faster than in the preparative run. The prolongation of step 4 is clearly evident, due to carry-over of threonine from the preparative run.

In Fig. 8 are reported the comparative assays of three fractions from the preparative run recorded in Fig. 5 and carried out at pH 8.1. Fraction 29 of zone I contained albumin, α_1-antitrypsin, and ceruloplasmin, and travelled in the Tachophor

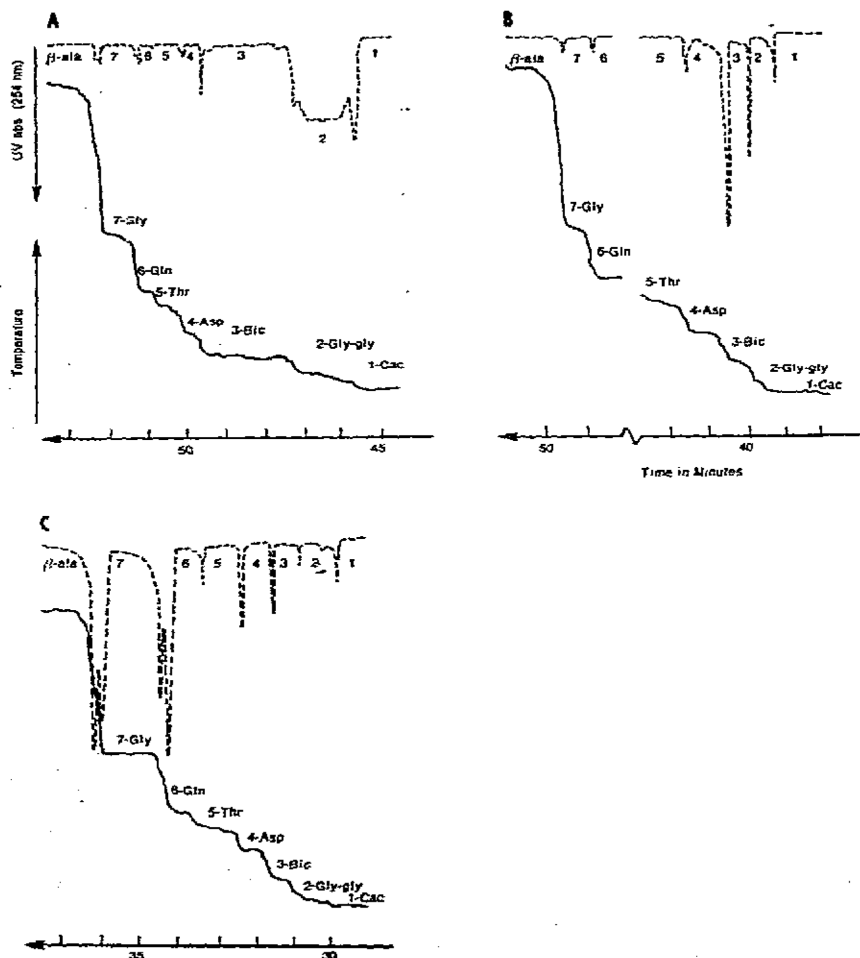

Fig. 8. Analysis with the Tachophor of the fractions obtained in experiment 5 (Fig. 5). Samples of 20 μl each were analyzed in the presence of 1 μl of the same spacers as reported in Fig. 7. The leading ion was 5 m*M* cacodylate at pH 8.1, and the data are presented as in Fig. 7. (A) Fraction 29, zone I; (B) Fraction 40, zone II; (C) fraction 48, zone III.

as a mixed zone with glycylglycine. Fraction 40, zone II, containing mainly hemopexin, haptoglobin, α_2-macroglobulin, and transferrin, formed two significant protein zones, separated by bicine. The proteins present in fraction 48, zone III, and containing IgA, IgG, and plasminogen, formed two major bands in free solution in the Tachophor, separated by glycine. Thus, in both of the experiments reported in Figs. 7 and 8, there was good qualitative agreement between the results of preparative and analytical ITP.

DISCUSSION

The potential appeal of ITP for preparative protein fractionation is the high concentration of proteins in the Kohlrausch adjusted zones. Unfortunately, the separated zones remain contiguous, there being no background buffer to separate protein components of different mobilities. It is, therefore, next to impossible to recover separately the contiguous zones without the use of some kind of spacer molecules having intermediate mobilities. Traditionally, in ITP, Ampholine was used for this purpose, since it contains a mixture of molecular species with a near continuous spectrum of mobilities. Unavoidably, mixed Ampholine–protein zones are formed which cause an indiscriminate lengthening of the protein zones not conducive to sharp separation between protein species of different mobilities. Vestermark[2] was the first to show that amino acids have net mobilities comparable to those of proteins, and may be used as discrete spacers in analytical ITP.

We have recently confirmed and extended these observations of Vestermark to preparative protein ITP on polyacrylamide gels[1]. Provided the number of spacers is not too large, n amino acids were found to produce $n+1$ sharply defined protein zones, both in free solution in the Tachophor, and on the gel. The majority of the ten specific proteins assayed were sharply resolved according to their mobility, this providing the best demonstration that ITP is capable of a high degree of resolution of complex protein mixtures. Nevertheless, ceruloplasmin and haptoglobin were not properly resolved, and were found to be present in more than one of the protein subgroups.

In the present work we have replaced polyacrylamide with Sephadex G-200, because of the greater ease of column preparation and absence of boundary anomalies. The results with both supporting media are rather comparable, except that the effective net mobility of the amino acid spacers is lower in Sephadex than in polyacrylamide, presumably because of the molecular sieving effect of Sephadex. Thus, with the leading electrolyte of pH 7.1–7.2, threonine separated albumin from the bulk of the globulins on polyacrylamide, while it barely migrated in front of the slowest proteins, IgA and plasminogen, on Sephadex (*cf.* Fig. 2). To effectuate separation of albumin from the bulk of the globulins on Sephadex, the much faster glycylglycine or bicine had to be used.

It was hoped that Sephadex will provide better resolution of all proteins than polyacrylamide due to the absence of boundary anomalies. This was not the case. With Sephadex, at pH 7.1, ceruloplasmin was found both in the first and in the second major protein zones, which is only marginally better than on polyacrylamide, where ceruloplasmin was found in all three protein zones. On Sephadex, haptoglobin was sharply confined to the second zone, while on polyacrylamide it was evenly

divided between the second and third zones. α_1-Antitrypsin, however, was now present in the first two peaks, while on polyacrylamide it migrated clearly with albumin only. Complete resolution of all proteins was not obtained in any of the systems tried.

We are compelled to conclude, therefore, that despite the exquisite flatness and sharpness of boundaries on Sephadex, the resolution of proteins in preparative ITP is not as good as one would wish or could anticipate from the theory of ITP, or its success in analytical separations of ions of low molecular weight. The sharpness of resolution of most proteins excludes gross experimental error, though some of the trailing is probably due to the elution system employed, or insufficient column length for complete resolution. Neither of these factors can explain, however, the bimodal distribution of some proteins within two spacer defined protein zones, such as the distribution of ceruloplasmin recorded in Fig. 2. This must be due to protein–protein interaction, favored by the high protein concentration characteristic of ITP. The well known electrophoretic heterogeneity of the immunoproteins IgA and IgG also surely contributes to their spreading.

Preparative ITP with discrete spacers is nevertheless a versatile new method for protein fractionation. Ideally, two spacers should be sufficient to bracket and isolate the desired protein mobility subgroup. It might be possible to subfractionate such subgroups taking advantage of changes in spacing characteristics of amino acids as a function of leader pH or the nature of the supporting medium. These and other possibilities are presently being explored for the specific isolation of some of the biologically important trace components of human plasma.

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