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SERUM PROTEIN FRACTIONATION BY ISOTACHOPHORESIS USING AMINO ACID SPACERS

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SUMMARY

Analytical and preparative isotachophoresis has been carried out using amino acids and peptides as discrete spacers in contrast to the usually employed continuous mobility spectrum Ampholine. Analytical isotachoresis in free solution, using the LKB Tachophor, demonstrated the separation of human serum into distinct mobility subgroups, *n* spacers giving rise to $n + 1$ protein subgroups. Preparative fractionation on polyacrylamide gel was carried out on the LKB Uniphor using threonine and glycine as spacers. Immunoelectrophoretic analysis showed that eight out of ten proteins assayed were clearly resolved in the three subgroups obtained, thus demonstrating the sharpness of isotachophoretic resolution.

INTRODUCTION

Isotachophoresis (ITP) is a relatively new variant of electrophoresis characterized by the use of a discontinuous buffer system, the sample being introduced at the interface formed by the so-called leading and terminating electrolytes[1–3]. The leading electrolyte should have co-ions with a net mobility higher than those of the sample ions, while the terminating electrolyte co-ions should have lower net mobility, there being a common counter-ion throughout the system. Under the influence of an electric field the sample ions separate according to their net mobilities into zones with sharp and self-stabilizing boundaries. Once steady state is achieved, all zones migrate at equal velocity, *i.e.*, the velocity of the leading ions, owing to the stepwise increase in field strength from zone to zone, as first described by Kohlrausch[4]. The concentration in each zone is independent of the concentration of sample applied, and adjusts itself to a function of the leading ion concentration, as also shown by Kohlrausch.

The technique is suitable for both analytical and preparative purposes, and has been applied to the separation of low-molecular-weight components[5–11], proteins[2,3,12–19], and even living cells[20].

For preparative protein separations, a unique appeal of ITP is the high concentration of the Kohlrausch-adjusted components achieved even at low concentrations of leader buffer. Since in ITP the separated zones are contiguous, as there is

no background buffer to fill the gap between molecular species of different mobilities, it is desirable to introduce spacer molecules of intermediate mobilities. Ampholine (trademark of LKB Produkter, Bromma, Sweden) is usually used for this purpose, as it provides an essentially continuous spectrum of mobilities within the mobility region of the proteins[12]. Spacing of relatively simple systems as, for example, artificial protein mixtures[13,14] is readily obtained. With more complex mixtures, such as human serum, many of the components are present in too small quantities to form distinct zones even in presence of Ampholine. Physical isolation of separated zones is further complicated by the frequently occurring distortions in the theoretically flat ITP boundaries in polyacrylamide gels and the partial re-mixing inevitable during the elution process. As a result, relatively poor resolution was obtained on analysis of effluents from polyacrylamide gel columns[3,12,17]. Nevertheless, ITP appears to be capable of high resolution as shown, for example, by complex patterns of hemoglobin obtained both in free solution and in gels[3,15,16], or the reported purifications of cholinesterase[18] and orosomucoid[12].

From a theoretical point of view, the degree of resolution obtainable by ITP is complicated by the simultaneous presence of field strength, temperature and pH gradients across each ITP boundary. The theoretical shape and thickness of interzonal boundaries in ITP has recently been calculated for simple ionic species, but such calculations for proteins are not available, owing to the complexity of their dissociation characteristics[21]. The use of Ampholine may further complicate the interpretation of results, as mixed Ampholine–protein steps are formed. In one mobility region there may be an excess of Ampholine, and in another proteins may be unresolved, owing to lack of sufficient spacing. In any case, the addition of Ampholine results in protein dilution, and necessitates the eventual separation of isolated proteins from this spacer. Thus, alternative methods for protein spacing are called for.

The purpose of the present paper was to investigate the use of amino acids as discrete spacers. It was first shown by Vestermark[19] that amino acids have mobilities similar to those of serum proteins, and could be used for this purpose. The use of discrete spacers has several advantages: ideally a mixture of n spacers with well defined mobilities should separate a complex protein mixture into $n + 1$ protein subfractions with no overlap or cross contamination across the spacer zones and no dilution of components. Thus, a judicious choice of two such spacers should permit the bracketing of the desired protein only, without causing any dilution of its or other protein zones. As the spacer zones can be made as long as desired, elution is facilitated. This approach has permitted a definitive determination of the sharpness of resolution obtainable by ITP, as will be reported.

MATERIALS AND METHODS

Reagents

Cacodylic acid, tris(hydroxymethyl)aminoethane (Tris), barium hydroxide and sucrose were obtained from Fisher Scientific (Pittsburgh, Pa., U.S.A.), N,N,N′,N′-tetramethylethylenediamine (TEMED) from Canalco (Rockville, Md., U.S.A.), hydroxypropylmethylcellulose (viscosity 15000 cP) from Dow Chemical (Midland, Mich., U.S.A.) (Methocel K 15M Premium), acrylamide and N,N′-methylenebisacrylamide from Eastman-Kodak (Rochester, N.Y., U.S.A.), riboflavin and amino

acids from Sigma (St. Louis, Mo., U.S.A.), glycylglycine from BDH (Poole, Great Britain), Ampholine from LKB Produkter, agarose (Indubiose A45) from L'Industrie Biologique Française (Gennevilliers, France) and human antisera (rabbit) from Behring Diagnostics (Somerville, N.J., U.S.A.).

Samples

Freshly drawn human serum was employed, as well as two crude fractions of serum. Fraction 1 was obtained by precipitation with 50% saturated ammonium sulfate. It contained less albumin than serum. An excess of albumin in the sample overshadows the detection of minor serum components.

Fraction 2 was obtained by precipitating the supernatant of fraction 1 at 75% saturation of ammonium sulfate, and contained mainly albumin. Both were dialyzed against the leader buffer, and recovered at a concentration of about 4% protein for fraction 1 and 2.5% for fraction 2.

Analytical ITP in free solution

The LKB 2127 Tachophor instrument was used with its ultraviolet (UV) and thermal detectors. Separations were performed in free solutions using a 0.4% solution of hydroxypropylmethylcellulose as stabilizer, and were carried out at 20° in PTFE capillaries, 0.5 mm I.D. and 24 or 36 cm in length. Mostly, only the UV detector (254 or 280 nm) was used, its resolution being higher. The leading buffer was 5 m*M* cacodylic acid, adjusted to pH 7.4–7.5 with 1 m*M* Tris, after addition of 0.6 ml of TEMED per liter buffer. TEMED was added to preserve similarity with ITP in gels, where it is necessary to achieve polymerization. The terminating electrolyte was 5 m*M* β-alanine, brought to pH 9.2 with barium hydroxide. Prior experience has shown that omitting the Tris counter-ion from the terminator does not affect the results, while barium hydroxide is needed to precipitate bicarbonate ions.

Preparative ITP in polyacrylamide gels

The separations were performed in the LKB Uniphor apparatus using the 40-cm glass column, thermostated at 12°. The gel length was 25 cm and contained the leading electrolyte, *i.e.*, 20 m*M* cadodylic acid with 1.25 ml TEMED per liter and sufficient Tris to bring it to pH 7.2. The gel concentration was 5% with 3% cross-linking and it was photopolymerized using 5×10^{-4} % riboflavin. The terminating electrolyte was 20 m*M* β-alanine and 10 m*M* Tris with barium hydroxide added to pH 9.2. The sample was applied at the boundary between the leader gel and the supernatant terminator, its layering having been facilitated by the addition of 10% sucrose. To avoid convection the sample was allowed to migrate into the gel at an initial voltage of 600 V, which was increased to 1200 V for the duration of the run. The amperage decreased from 15 to 8 mA, and the total separation time including elution was 26 h. The leading electrolyte was used as the elution buffer, at a flow-rate of 15 ml/h. An approximate calculation indicated a three-fold dilution of the zones due to elution. The UV absorbance of the effluent was recorded using the LKB 8300 Uvicord II. A total of forty 2-ml fractions were collected.

Immunoelectrophoretic analysis

The protein fractions collected from the Uniphor were analyzed using a modi-

fied Laurell technique[22]. Pre-coated glass slides, 8 × 10 cm, were layered with 10 ml of 0.85% agarose, buffered to pH 7.4 with a 5 m*M* HCl–Tris buffer. Most runs were carried out with the incorporation of only 0.1 ml of monospecific anti-serum per 10 ml agarose, corresponding to 10% of the concentration suggested in the original method. For more dilute samples as little as 0.02 ml antiserum per 10 ml of agarose permitted good rocket formation. Calibration curves were established by analyzing serum samples and serum fraction 1 at various dilutions, and the data were reported as percentage of each protein with reference to the original serum fraction 1.

Amino acid analysis

Quantitative determination of amino acids in the fractions collected from the Uniphor was performed in the Beckman Automatic Amino Acid Analyzer Model 121 using the hydrolyzate procedure on samples deproteinized with sulfosalicylic acid.

RESULTS

The first object of this investigation was to confirm the possibility of using discrete spacers, *i.e.*, to find non-UV-absorbing ionic species within the mobility range of proteins, and prove that these molecules produce well defined zones between the stacked proteins. Amino acids and dipeptides were the most likely prospects in accordance with the original finding of Vestermark[19] and the LKB 2127 Tachophor the most suitable apparatus for this purpose. Only small sample quantities were required, of the order of 1 μl serum, and an analysis could be performed within 30 min. The apparatus is equipped with a high resolution UV detector (slit width 0.2 mm), which easily discriminates between the UV absorbing protein zones and the non-UV absorbing spacer zones as they migrate past the fixed detector.

Fig. 1A illustrates the closely stacked protein pattern formed by 1 μl of serum in absence of either Ampholine or any discrete spacers. One can obviously recognize a major peak, presumed to be albumin, and several globulin peaks, but the resolution is poor and assignment of individual peaks impossible, as it is to be expected in absence of any spacers. Fig. 1B shows the clear cut separation of the stacked proteins into five subgroups, resulting from the addition of four discrete spacers: glycylglycine, asparagine, threonine and glycine. The scale printed on the bottom of each pattern illustrates the length of each zone in the Tachophor capillary. The spacing caused by the addition of the individual amino acids is of the order of 1–2 cm, which is sufficient to permit elution of the protein subgroups if the same is reproduced on a preparative column.

Serum albumin is responsible for the biggest peak of Fig. 1, being the fastest serum component and accounting for about 60% of total protein. Thus, it overshadows the many fractions in the globulin range. In order to obtain a better proportion of globulins *versus* albumin in the starting material, the previously described fraction 1 was prepared by precipitation of serum at 50% ammonium sulfate. Its pattern, in presence of the four spacers, is presented in Fig. 2B, and is contrasted with the pattern in Fig. 2A obtained from fraction 2, which contains mostly albumin. This experiment confirmed that the spacing of the amino acids is not arbitrary, but that they do space the components of a sample according to their mobilities. The zones are arranged in order of decreasing mobilities from leader to terminator and it can be seen that frac-

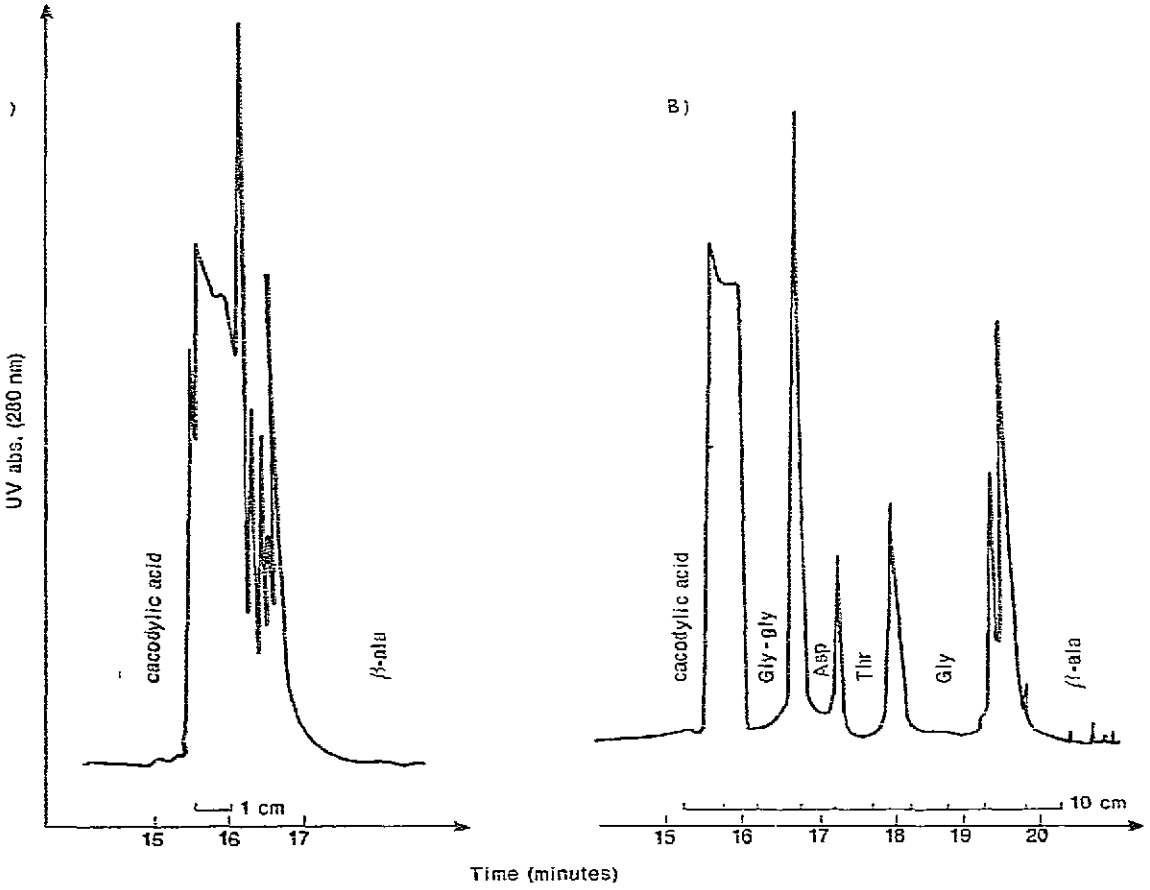

Fig. 1. Analysis in the Tachophor using a 24-cm-long capillary of (A) 1 μl serum and (B) 1 μl serum mixed with 1 μl of each of the four spacers: glycylglycine, asparagine, threonine and glycine (2 g/l each). Leading ion was 5 m*M* cacodylic acid (pH 7.5) and terminating ion was β-alanine (pH 9.2). Electrophoresis was performed at a constant current of 100 μA, the voltage increasing from 5 to 15 kV. The first sample appeared at the UV detector in 15 min.

tion 2 contains mostly proteins with high mobilities, *i.e.*, in the mobility range from that of cacodylic acid to asparagine, whereas fraction 1 is rich in components of low mobility (from threonine to β-alanine), roughly corresponding to albumin and globulins, respectively.

The protein content in the first, high-mobility, subgroup is noticeably different for the two fractions, not only in respect to quantity, but also heterogeneity. The third UV peak between asparagine and threonine appears, however, to be homogeneous. To obtain a true picture of heterogeneity of each subgroup, the addition of a continuous spectrum spacer, *i.e.*, Ampholine is necessary. This is exemplified by patterns of the same two fractions in presence of a small quantity of Ampholine (0.5 μl of a 1% solution) (Fig. 3). The previously homogeneous peak between asparagine and threonine has clearly separated into two peaks, and the total number of peaks in

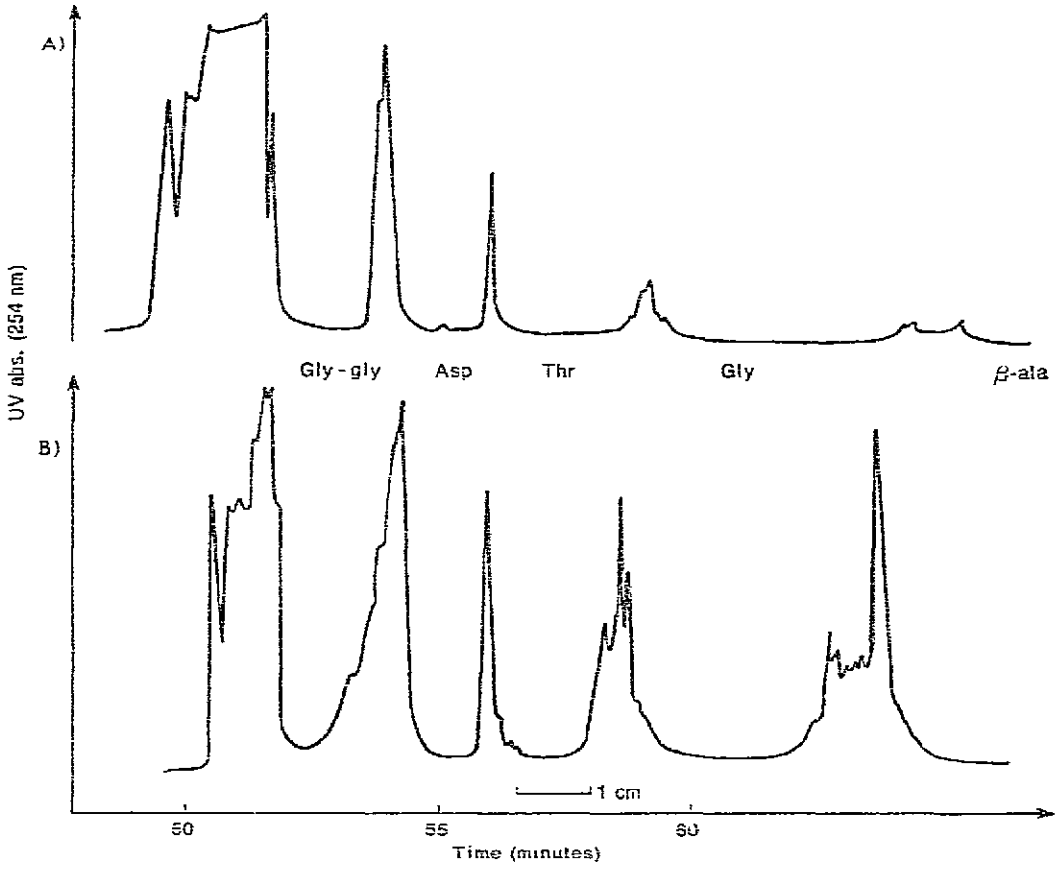

Fig. 2. Analysis in the Tachophor using a 36-cm-long capillary of (A) 1.25 μl of serum fraction 2 (2.5% protein) and (B) 0.75 μl of serum fraction 1 (4% protein) in the presence of the four individual spacers (each 1 μl, 2 g/l). The same buffers as in Fig. 1 were used, but analysis was carried out at 40 μA, and the voltage increased from 5 to 26 kV.

all the fractions becomes comparable to that obtainable with other high-resolution techniques such as isoelectric focusing or disc electrophoresis. The addition of Ampholine causes also an overall spread of the pattern as mixed protein–Ampholine zones are formed. To obtain complete separation of the still superimposed minor peaks greater amounts of Ampholine would be necessary.

Preparative ITP using polyacrylamide gels in the LKB Uniphor system was performed to demonstrate the usefulness of amino acids as discrete spacers on a larger scale. To prove this point two amino acids, threonine and glycine, were selected. As starting material the albumin-impoverished fraction 1 was used. A second objective of the experiment was to provide definitive evidence for the sharpness of isotachophoretic resolution of protein components. The column was therefore loaded with a small quantity of protein in proportion to amino acid concentration to provide relatively long zones of spacers, thus minimizing cross-contamination during elution. The sample consisted of 3 ml of serum fraction 1 (approx. 4% protein) and 3 ml each

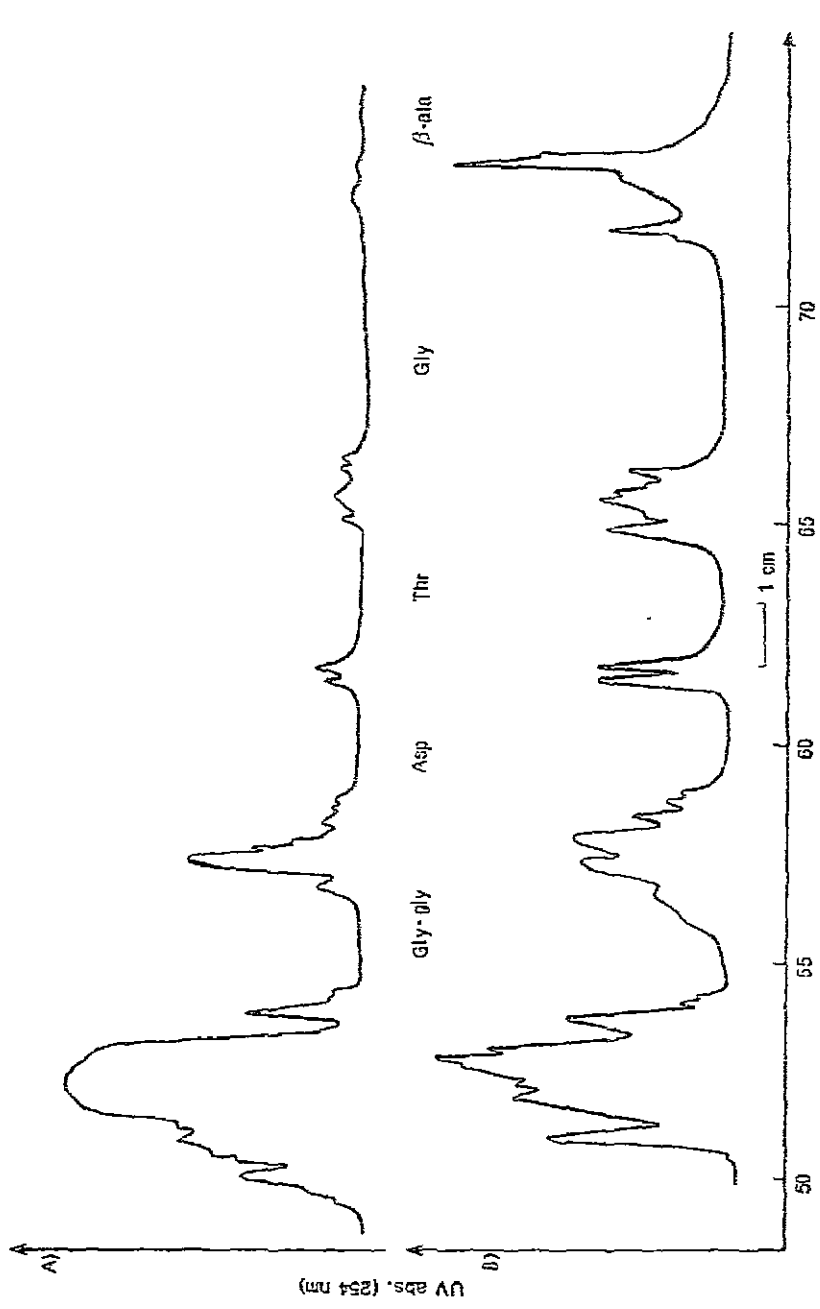

Fig. 3. Analysis in the Tachophor using a 36-cm-long capillary of (A) 1.25 μl of serum fraction 2 and (B) 0.75 μl of serum fraction 1 in presence of the four individual spacers (each 1 μl, 2 g/l) and 0.5 μl of 1% Ampholine (pH range 3–10). The same buffers as in Fig. 1 were used and the samples were detected at a constant current of 40 μA.

of threonine and glycine (6 g/l). The leading ion was 20 m*M* cacodylic acid and the terminator β-alanine, as previously described. Constant voltage of 1200 V was applied during most of the run, including elution. Visual inspection of the column after the protein zones were resolved showed a leading yellow disc-like zone of albumin and two similar reddish zones, probably due to traces of hemoglobin in the sample and its possible interaction with haptoglobin. This is illustrated in Fig. 4. Amino acid analysis showed that threonine was the spacer between albumin and the first reddish zone while glycine spaced the two reddish zones. The typical zone anomalies encountered in polyacrylamide gel ITP were seen as uneven hemoglobin concentration across the band.

The UV absorbance of the eluate reproduced in the top tracing of Fig. 5 shows the expected separation of the serum proteins into three major subgroups. A total of forty 2-ml fractions were obtained and analyzed by using our modification of the Laurell technique. Fig. 6 shows a typical Laurell slide used for the quantitation of individual proteins, in this case transferrin. The first four samples contained standard dilutions of serum, while the remaining seven samples comprised representative frac-

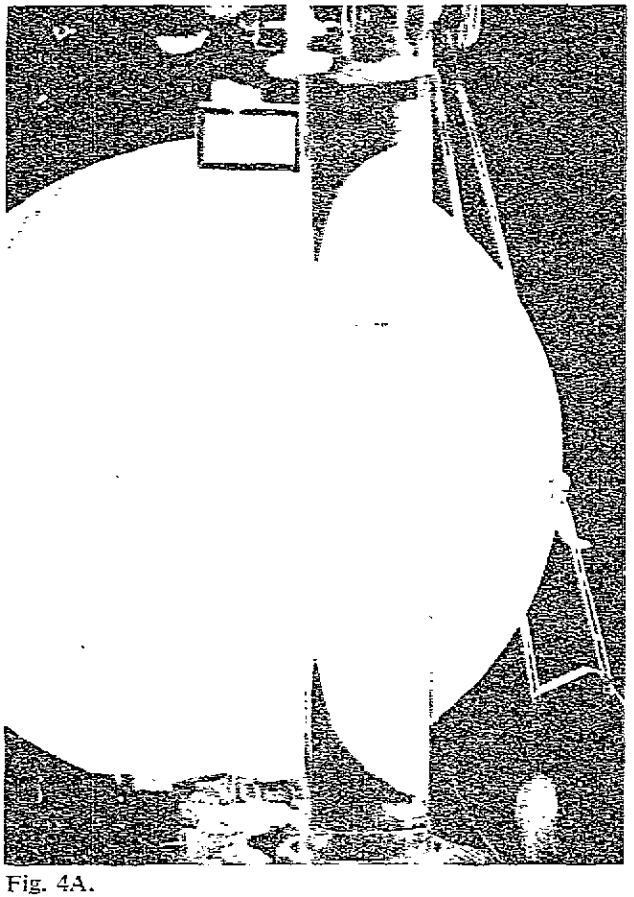

Fig. 4A.

tions of the eluate. The concentration of ten specific proteins, in terms of their relative concentration in column eluates, *versus* the concentration in original fraction I is plotted on the bottom of Fig. 5. Albumin and α_1-antitrypsin are present only in the first subgroup. Hemopexin and transferrin are located in the second group, and the third comprises IgA, IgG, plasminogen and α_2-macroglobulin, all of these showing rather sharp resolution between the subgroups. Difficult to explain, however, is the presence of ceruloplasmin in all three groups and haptoglobin in subgroups II and III. The sharpness of resolution of the other components excludes experimental error or cross-contamination during elution, and this finding can be tentatively ascribed to protein–protein interaction amplified by the high concentration of proteins in the zones.

Amino acid analysis using the Beckman Analyzer (Table I) shows excellent resolution of the individual amino acids confirming the presence of only threonine in the first spacing zone (fraction 21), glycine in the second (fraction 29), and β-

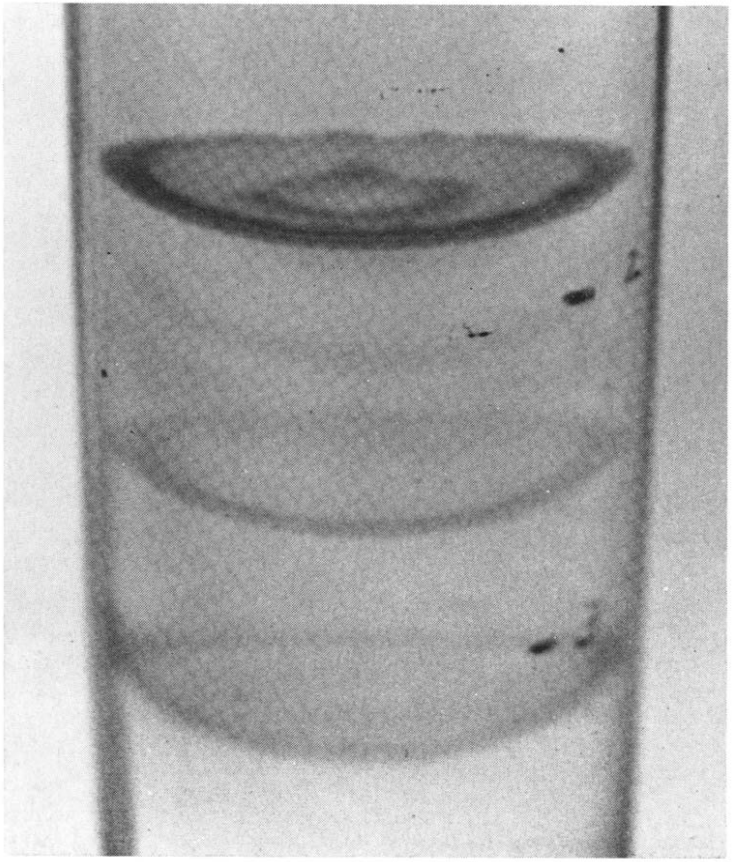

Fig. 4. Analysis in the Uniphor system of 3 ml of serum Fraction I (4% protein) in the presence of threonine and glycine (each 3 ml, 6 g/l). Three protein bands were visible: a yellow band from albumin and two reddish bands, presumably due to trace amounts of hemoglobin in the sample. (A) shows the sharpness of separated fractions; (B), taken from an angle, shows typical anomalies in the zone boundaries resulting in an uneven hemoglobin concentration across the band.

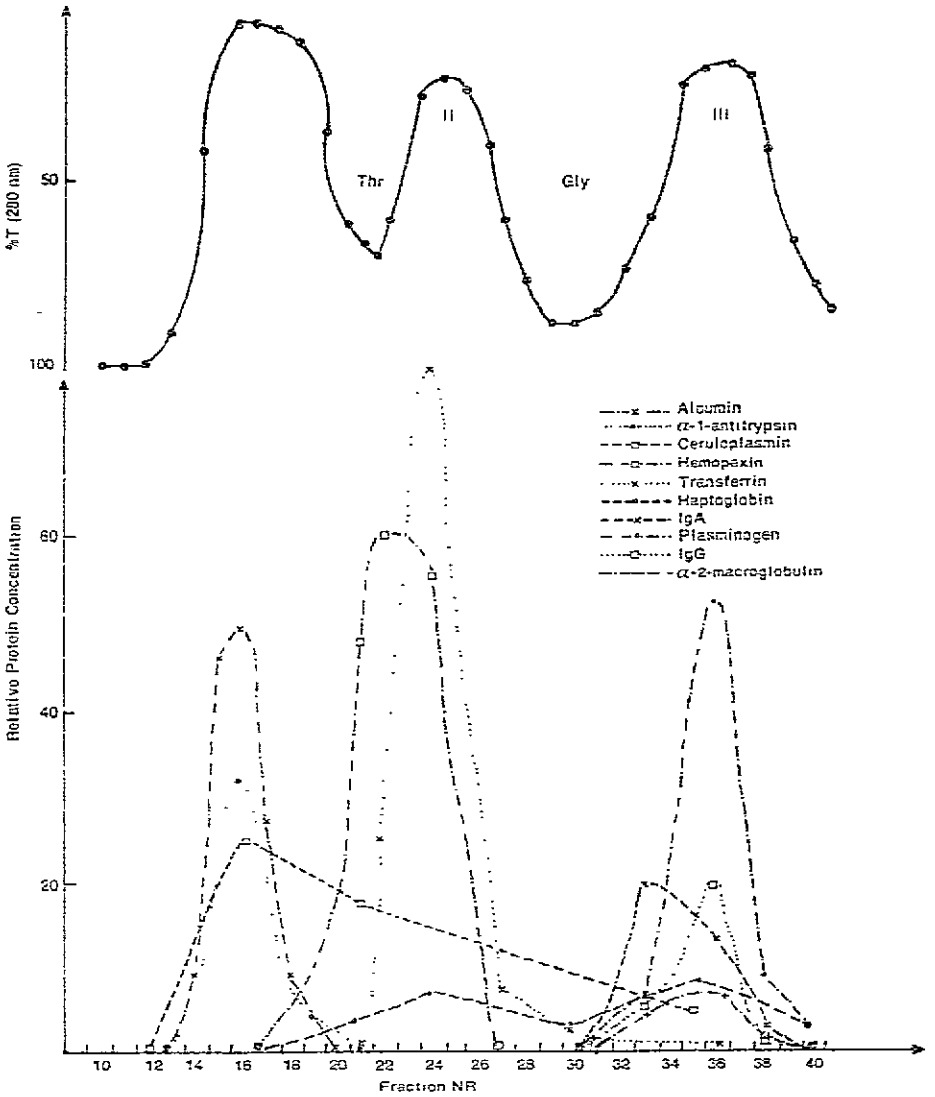

Fig. 5. Fractionation in the Uniphor system of 3 ml of serum fraction 1 in the presence of threonine and glycine. Separation was performed with 20 m*M* cacodylic acid as leading ion (pH 7.2) and *β*-alanine as terminator. During elution with leading electrolyte (15 ml/h) the current was 8 mA. The UV absorbance of the fraction was recorded. The analysis of ten specific proteins is seen below. The protein concentration is given as percentage of the concentration of the original sample, the serum fraction 1.

alanine in the terminator (fraction 40). The presence of threonine in the second protein subgroup (fraction 24) and of both glycine and *β*-alanine in the third protein subgroup (fraction 35) is probably due to mixing caused by the elution of the fractions from the column. In fact the sharpness of the protein peaks (Fig. 4) appears to be far greater than that indicated from the Uvicord recording (Fig. 5).

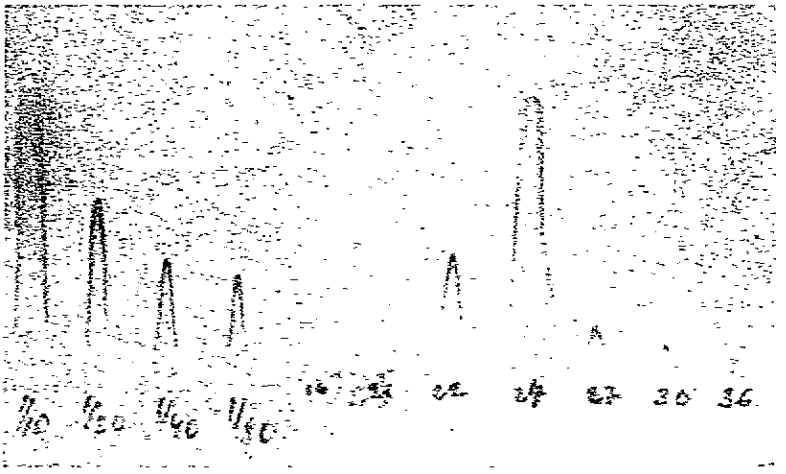

Fig. 6. Analysis of transferrin by the Laurell rocket technique. Reference samples of serum at 10-, 20-, 40- and 80-fold dilution were applied in the first four holes together with seven fractions collected from the Uniphor experiment at 1:1 dilution with leading electrolyte.

TABLE I

AMINO ACIDS ANALYSIS OF THE UNIPHOR FRACTIONS

— = Not detectable.

Fraction No.	*Threonine (mM)*	*Glycine (mM)*	*β-Alanine (mM)*
16	1.7	0.1	—
21	20.4	0.1	—
24	5.7	0.0	—
29	—	12.9	—
35	—	4.6	10.9
40	—	0.0	30.3

DISCUSSION

The use of the amino acids as specific spacers had a two-fold purpose: on the one hand, we wanted to test the hypothesis that ITP is indeed a high-resolution method. This would have required a clear separation of every protein into one of the three groups obtained by the interposition of the two amino acids. Using Ampholine, the minor components of serum are bound to be overlapping, as they do not form sufficiently long zones to be separated individually, unless a tremendous excess of Ampholine is used. Thus, only discrete spacers such as amino acids, can provide the definitive experiment to evaluate the sharpness of resolution of ITP.

On the other hand, the purpose of using discrete spacers is to eliminate some of the obvious drawbacks of Ampholine, namely the dilution of the protein zones, prolongation of separation time needed for the resolution of Ampholine, high cost of Ampholine, etc. It is true that discrete spacers cannot separate individually all the proteins, but one would hope that with the proper choice of amino acids or peptides, pH and other conditions, one could specify conditions isolating the desired protein in a subgroup of only one or few proteins at worst. This has been partly sub-

stantiated in our experiments and we have evidence that ITP does have the potential of sharp resolution. The failure of ceruloplasmin and haptoglobin to be clearly resolved is as yet puzzling and any explanation is bound to be hazardous.

We have demonstrated that ITP with the use of discrete spacers provides an excellent method for fractionation of complex proteins into broad subfractions and feel that this technique will be a significant addition to the currently available separation methods. Its main advantage is the large capacity of the columns, since the proteins travel in highly concentrated zones which after elution are similar in concentration to the originally applied sample. Although in the current experiment only 3 ml of a 4% protein sample was applied to the Uniphor, much larger volumes appear to be separable as judged by the sharpness of fractions shown in Fig. 4A. Furthermore, we have shown that the LKB Tachophor is a useful tool for preliminary screening of discrete spacers. The identification of Tachophor peaks is not yet possible and the sequence of individual proteins in free solution may be different than in gels. Nevertheless, the significantly different patterns obtained in the Tachophor for different protein mixtures serve to indicate both the mobility range and the heterogeneity of the samples and thus confirm the usefulness of analytical ITP.

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