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Isoelectric Separation of Urinary Bence-Jones Proteins and Light Chains of Human IgG*

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Summary

Urinary Bence–Jones proteins from three different patients and two preparations of light chains derived from normal, pooled human IgG either by oxidative sulfitolysis or reduction/alkylation were subjected to isoelectric separation in a vertical column containing a combined pH and density gradient. The Bence–Jones proteins could be shown to be heterogeneous in pH gradients covering 0.5 to 7 pH units. In narrow pH gradients the resolving power was improved. In pH gradients covering the range of pH 3–10 and in the presence of 5 *M* urea the light-chain preparations could be partially resolved into the bands well known from gel electrophoresis.

INTRODUCTION

Since moving-boundary electrophoresis was introduced by Tiselius various electrophoretic methods for the fractionation of proteins have been described. One of these techniques, electrophoresis in a pH gradient, separates ampholytes according to their isoelectric points. The general idea of isoelectric fractionation has been known for some time [Svensson (*1*)], but in regard to its present application the technical problems of obtaining a stable pH gradient and preventing convection had to be overcome. Starting in 1961, Svensson (*2–4*) published a series of papers on the theoretical and practical aspects of the

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method, and Vesterberg and Svensson (*5*) developed a method for synthesizing low-molecular-weight carrier ampholytes suitable for producing stable, natural [Svensson (*2*)] pH gradients. Kolin (*6*) succeeded in preventing convective disturbances in vertical liquid columns by using sucrose density gradients.

The present status of these developments is a method that, in a combined pH and density gradient, allows the focusing of high-molecular-weight ampholytes such as proteins into isoelectric zones [Vesterberg and Svensson (*5*)]. At the same time the isoelectric points (IEP) of the resolved components can be determined [Svensson (*2*)].

In order to test its potentialities it was decided to apply this method to the separation of urinary Bence–Jones proteins (BJP). Because of reasonable results with BJP it was obvious to apply the same method to light chains of immunglobulins.

MATERIALS AND METHODS

Bence–Jones Proteins

From a large choice of urine specimens from different myeloma patients three samples (Gr, Du, Fi) were selected that showed essentially one component in paper electrophoresis. Proteins from 50–100 ml of urine containing 2–10 mg of protein/ml were precipitated by stepwise addition of solid ammonium sulfate at pH 7.0 until samples of the supernatants remained clear after acidification with a 10% solution of trichloroacetic acid. The precipitates were dissolved in a minimal amount of water, freed of insoluble material by centrifugation, desalted by passing through a column containing at least five times the sample volume of Sephadex G-25, and eluted with 0.05 *M* imidazol/HCl buffer pH 7.0. To remove residual urinary pigments 5 ml portions of effluent from Sephadex G-25 containing 20–80 mg of protein were chromatographed on 1.4 × 35-cm columns of DEAE-Sephadex A-50 equilibrated with 0.05 *M* imidazol/HCl buffer pH 7.0. All proteins from urine Gr and Du could be eluted with this buffer. From urine Fi no proteins did pass the column under these conditions, but could be eluted with 0.1 *M* NaCl by gradient elution at constant pH using 1 liter of 0.05 *M* imidazol/HCl buffer pH 7.0 in a constant-volume mixing bottle and the same buffer containing 1 *M* NaCl in the reservoir. In all three cases colored material was held back at the top of the column, and heterogeneity of eluted protein was indicated by the unsymmetrical shape of $O.D._{280m\mu}$-curves.

Effluents were concentrated to a protein content of 0.2–2% by pressure dialysis against a 1% solution of glycine.

As judged by comparison of cellulose acetate electrophoresis of native urine and final protein solution, no essential protein fraction was lost during this purification.

Light Chains of IgG

Pooled human IgG from Cohn fraction II was further purified by passing through a column of DEAE-Sephadex A-50 equilibrated with 0.05 *M* imidazol/HCl buffer pH 7.0. Elution was carried out with the same buffer. IgG prepared in such a manner showed one single line when tested in immunoelectrophoresis with an antiserum against complete human serum.

Cleavage of disulfide bonds was accomplished by limited oxidative sulfitolysis [Franěk and Zikán (*7*)] or by reduction with 0.2 *M* 2-mercaptoethanol and alkylation with 0.3 *M* iodoacetamide essentially after Fleischman, Porter, and Press (*8*). In every case excess reagents were removed by gel filtration on Sephadex G-25 in 0.2% ammonium carbonate and the eluate containing the protein was freeze-dried. Separation of polypeptide chains was realized by gel filtration of 400 mg of lyophilized protein, dissolved in 20 ml 5 *M* urea/0.05 *M* formic acid on a 5.8 × 75-cm column of Sephadex G-100 equilibrated with the same solvent.

Fractions from the light-chain peak were pooled, passed through a column of Sephadex G-25 in 0.2% ammonium carbonate, and lyophilized. A sample of this light-chain preparation, dissolved in 5 *M* urea, was tested in immunoelectrophoresis with an antiserum against Fc fragment of human IgG. No contamination with heavy chains could be detected, but precipitation occurred with antisera against BJP k and λ.

Disc Electrophoresis

BJP were separated in 7.5% acrylamide gels at pH 9 [Davis (*9*)]. Before electrophoresis, proteins were dialyzed against large-pore gel buffer pH 6.7 overnight, diluted to correspond to the actual buffer concentration in the gel. 0.05–0.1-ml samples containing 5–30 μg of protein and 10–20% of sucrose were applied without polymerization on top of the large-pore gel.

Separation of light chains was performed at pH 9.4 in 4% acryla-

mide gels containing 10 *M* urea [Reisfeld and Small (*10*)]. Upper-tray electrode buffer was used in both upper and lower trays and no urea was added. Proteins were dialyzed against a solution containing large-pore gel buffer diluted as indicated for BJP but containing urea to make a 5 *M* solution. Samples of 5–30 μg of protein in 0.1 ml were layered without polymerization on top of the large-pore gel.

During the first 15 min of electrophoresis, current was held at 2 mA/tube and did not ever exceed 5 mA during separation.

Proteins were fixed and stained by immersion for at least 24 hours in a solution composed of 125 mg of Coomassie Brilliant Blue R per liter of a 40% methanol/5% acetic acid solution [Uriel (*11*)]. Gels were destained by leaching in 7% acetic acid.

To detect possible loss of protein during dialysis, disc electrophoresis of undialyzed fractions was carried out. Although the carrier ampholytes present did affect the separation because of their relatively large concentration and pH variation, the comparison showed that qualitatively no proteins were lost. The carrier ampholytes could be stained like proteins and did migrate in bulk just behind the buffer front.

Protein Content

Concentration of BJP and light chains was determined by UV absorption by using a specific extinction coefficient $E^{1\%}_{1cm} = 13.5$.

Isoelectric Separation

Electrofocusing column and carrier ampholytes available commercially from LKB* were used. The separation compartment of the column had a volume of 110 ml; carrier ampholytes were obtained as water solutions with a dry content of 40% *w/w* and covering either the large range pH 3–10 or a small range of 2 pH units between pH 3–10. A temperature-controlled 10-liter water bath with a circulating pump was used to keep the desired temperature within ±0.2°C.

The electrofocusing experiments were carried out according to the method described by Vesterberg and Svensson (*5*), briefly as follows:

A density gradient was built up in the column by the use of a dense and a less dense solution. The dense solution was prepared by dissolving 28 g of sucrose and three-fourths of the total amount of carrier ampholytes in water to a volume of 60 ml; the less dense solution

* LKB-Produkter AB, Stockholm-Bromma 1, Sweden.

contained one-fourth of the ampholytes in the same volume. The higher concentration of ampholytes in the dense solution was used to balance the higher viscosity in this solution, tending to decrease the conductance. The total amount of carrier ampholytes was 1.2 g, except for one experiment in which 4 g were used. The two solutions were transferred to burets. The dense solution was distributed into 24 test tubes, beginning with 4.6 ml in tube 1, decreasing linearly to zero in tube 24, and the volume in each tube completed to 4.6 ml with the less dense solution. In one of the middle fractions the less dense solution was replaced by sample solution. Samples contained 5–10 mg of protein in 0.2–2 ml of a 1% glycine solution. The bottom electrode solution contained 60% *w/v* sucrose and 2% ethylene diamine or 1% phosphoric acid, depending whether the cathode or the anode was at the bottom. This solution was first introduced into the vertically mounted column and subsequently the 24 fractions were piled up in their number order. Finally, the complementary top electrode solution, 2% ethylene diamine or 1% phosphoric acid, was added on top. In some experiments all solutions contained 5 *M* urea in addition. Separation was started by applying a potential of 300 V for the pH range 3–10 and up to 700 V for narrower gradients. Whenever necessary voltage was increased stepwise to avoid electrical loads higher than 1 W.

The final steady state, being an equilibrium between electrophoretic migration and diffusion of proteins and carrier ampholytes, was indicated by constant current.

Before taking fractions, the current was interrupted and the lower electrode compartment closed by a special locking device to avoid distortion of the pH gradient by the alkaline or acid electrode solution.

At the beginning, fractions were taken manually from the outlet tube at the bottom of the column, and the flow rate was controlled with a pinch clamp. After a few experiments, however, the draining of the column was regulated with a peristaltic pump attached to the bottom tubing, and fractions were collected mechanically after passing through a uv monitor/recorder. The flow rate was adjusted to 1 ml/min and fraction volumes were kept between 2–2.5 ml.

The temperature was held constant throughout the whole experiment by passing water from the thermostat through the cooling mantle of the column. All experiments with BJP were done at 6°C; light chains were separated at 24°C.

The pH of each fraction was determined with a pH meter on which

repeated measurements could be reproduced within ±0.05 pH units. The calibration and the measurements were carried out at 20–25°C.

According to Svensson (*2*) the IEP of the resolved components can be determined by simply measuring the pH at the point of maximum concentration. The IEP at 25°C can be obtained by making the pH measurement at this temperature, irrespective of the temperature during isoelectric separation [Vesterberg and Svensson (*5*)]. The values refer to essentially zero ionic strength because salts migrate to the electrodes.

RESULTS

Isoelectric Separation of BJP in the Screening Range pH 3–10

The electrophoretic mobilities of the three BJP at pH 8.6 on paper, compared with serum proteins, were roughly γ (Gr), β (Du), α (Fi). The same sequence of mobilities was obtained in disc electrophoresis (Fig. 1). Hence IEP ranging approximately from 8 to 5 could be ex-

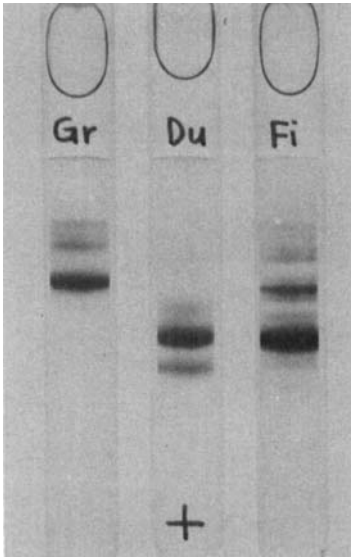

FIG. 1. Disc electrophoresis of urinary BJP after chromatography on DEAE-Sephadex. The amount of protein was 20 μg (Gr, Du) and 30 μg (Fi), each in 0.05 ml. Conditions: 2 h (Gr, Du) and 1 h (Fi) at 4–5 mA/tube and 200 V. The migration of the protein was from cathode toward anode.

pected. For the first few experiments the widest pH range (pH 3–10) was therefore selected (Fig. 2). Voltage was set at 300 V from the beginning and held constant. Initial current was between 3 and 4 mA, declined rapidly within the first few hours, and finally remained at a constant value of 0.5 mA at the latest after 40 hr. After a total of 46.5 to 48 hr the current was turned off and fractionation started.

During isoelectric separation, precipitation at various positions in the column occurred. In the case of BJP Gr, e.g., 6 hr after the current was turned on a faint turbidity could be seen at the interface between the cathode solution and the lowest density-gradient fraction. Two bands, 3 and 5 mm wide, moved out of the upper electrode compartment after one day. When the current was turned off after 48 hr, turbidity in the lower electrode solution was unchanged and the two bands had sedimented to the position indicated by arrows in Fig. 2. The material in the lower one of the two bands was flocculated and a few single particles were visible all the way down the column. The electrode solutions were of a light yellow color. In the experiments with BJP Du and Fi, precipitates appeared in the same manner within the first day, but intensity and final position of the bands varied. The fractions marked by an arrow were cleared by filtration through a millipore filter (pore size 0.8 μ). In the filtrate practically no proteins could be detected, except for BJP Fi. In this case (Fig. 2c) precipitation bands and the main peak of soluble proteins were superimposed and therefore separation obscured. To overcome this difficulty BJP Fi was separated once more with electrode positions reversed (Fig. 2d). Under these conditions precipitation occurred only at the anode compartment in the bottom of the column.

Disc electrophoresis with combined fractions 14, 15, 16 (Fig. 2a) and 11, 12, 13 (Fig. 2d) showed exactly the pattern of the starting materials BJP Gr and BJP Fi (Fig. 1) and 90–95% of the applied proteins were found in fractions 13–19 from Gr and in fractions 10–15 from Fi. Rough estimates of the IEP were 7.8 and 4.6 for BJP Gr and Fi, respectively.

Disc electrophoresis on single fractions 19–27 (Fig. 2b) showed definitely some resolution of BJP Du (Fig. 3a). Practically all proteins were located within a range of pH 5–7.

Isoelectric Separation of BJP Du in Narrow pH Gradients

Local concentration of focused proteins becomes lower in a narrower pH gradient because of the widened zone in a shallow gradient.

Larger samples of protein can be applied to the column. In the following experiments the amount of protein was increased to 10 mg. Figure 4a shows the separation of BJP Du in a gradient covering the range of pH 5–7. To avoid precipitation at the top of the column, electrode positions were reversed in contrast to Fig. 2b. To get the same sequence of separation pattern the fraction numbering was begun at the right side of the graph. Voltage was set at 300 V at the begin-

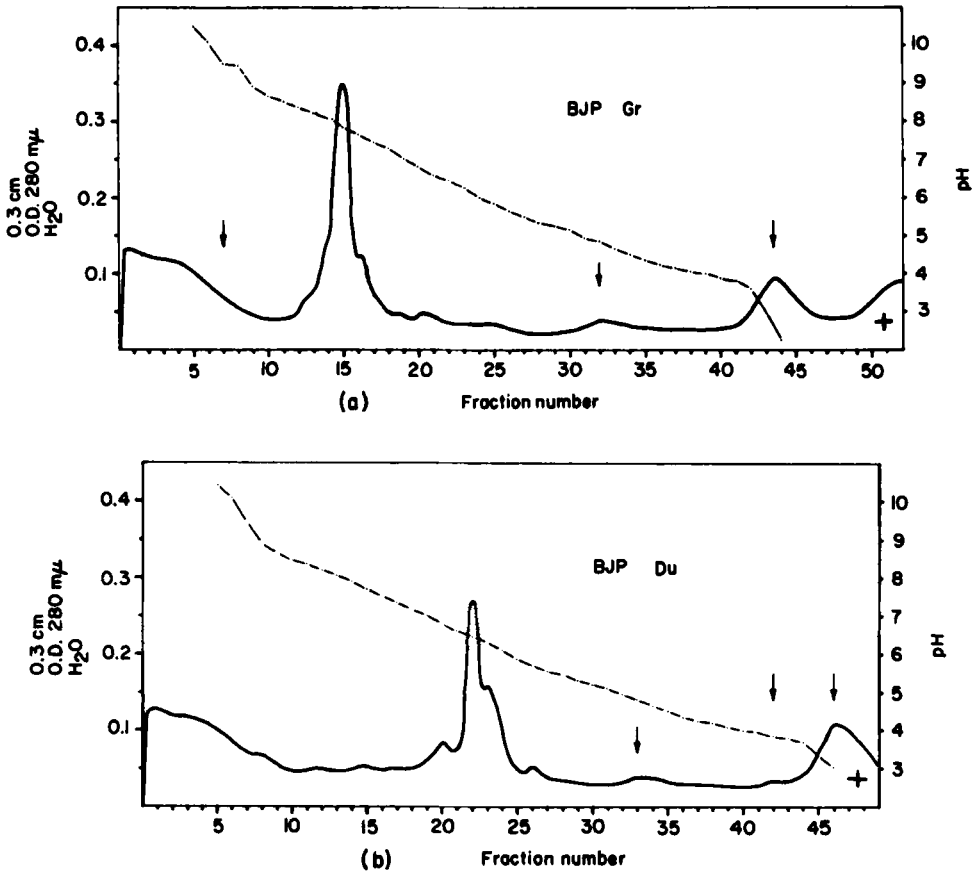

FIG. 2. Isoelectric separation experiments on three BJP samples in pH gradient 3–10. In each run 5 mg of protein was used. In a, b, and c the anode was on top of the column, in d at the bottom. The positions of the precipitates at the end of the run are indicated by arrows. In all graphs approximately the first ten and the last ten fractions contain upper or lower electrode solutions. —·— pH —— O.D.

ning and then raised stepwise up to a constant value of 700 V within 2 hr in such a way that the electrical load did not exceed 1 W. The current dropped to a constant value of 0.9 mA within 30 hr and fractionation was started after a total of 49 hr. No visible precipitates appeared outside the electrode compartments. Disc electrophoresis on combined and single fractions are shown in Fig. 3b. Figure 4b shows the result of a run without proteins. Experimental conditions including carrier ampholytes were the same as for the experiment shown in Fig. 4a. The anode solution at the bottom of the column was slightly turbid and yellow.

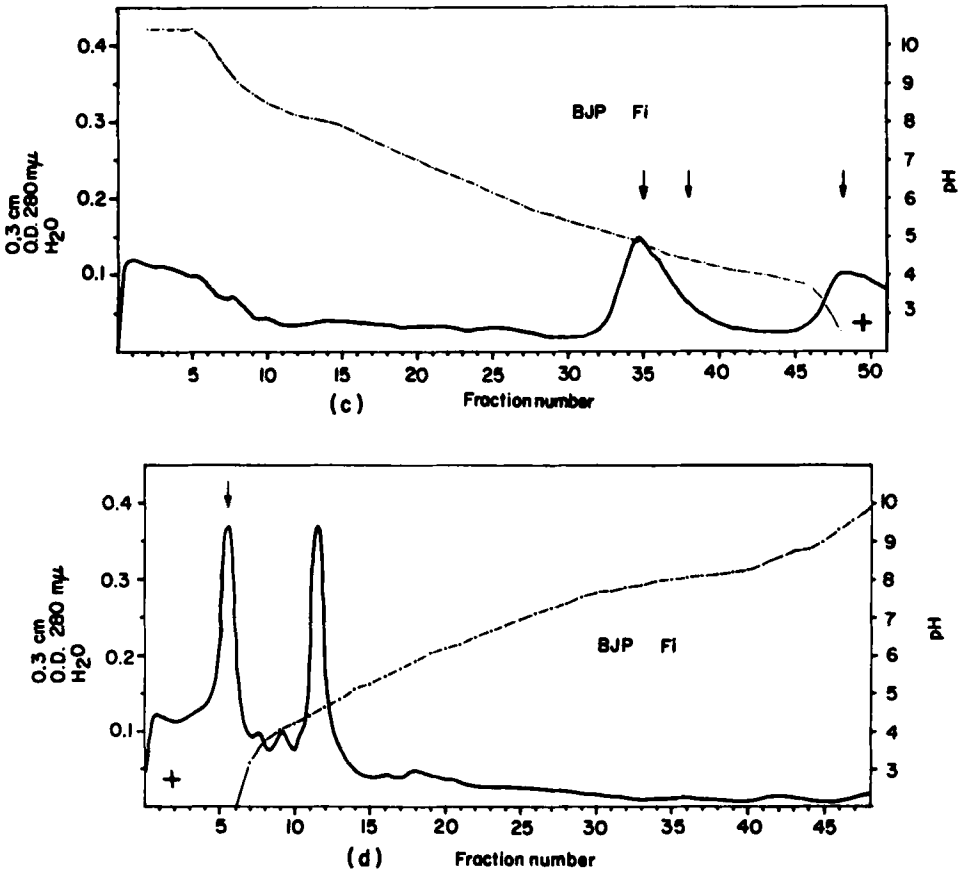

FIG. 2 (continued).

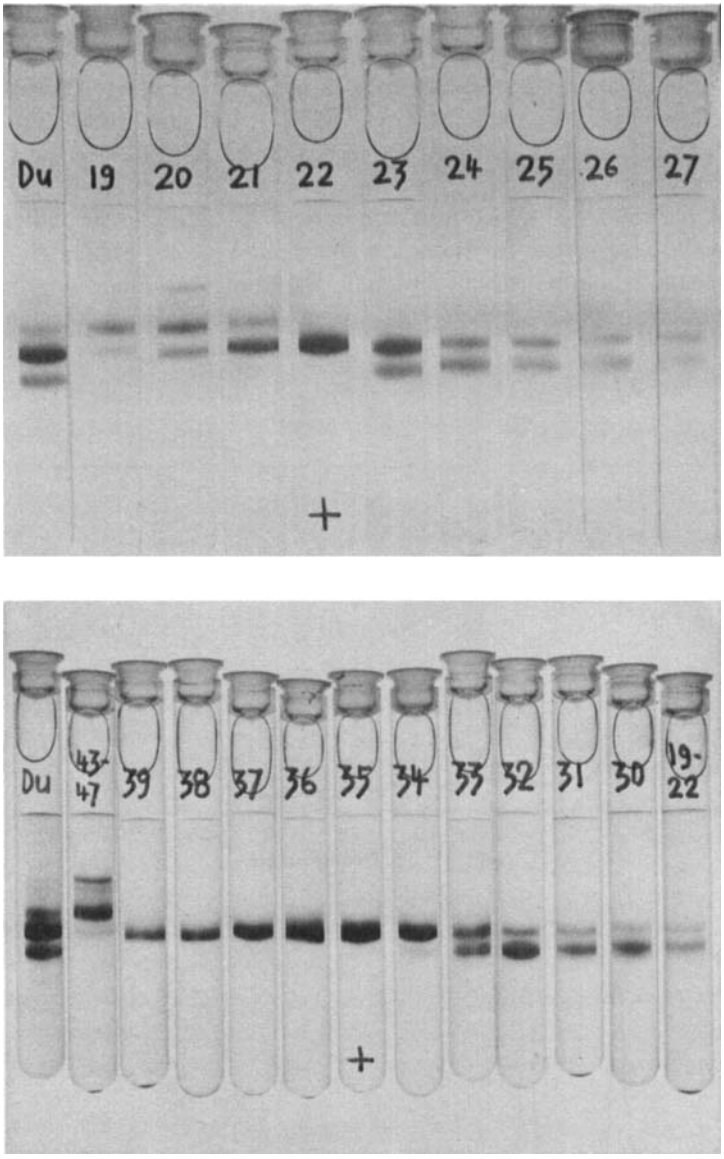

FIG. 3. Disc electrophoresis of BJP Du after isoelectric separation in various pH gradients: (a) fractions from pH gradient 3–10 (Fig. 2b); (b) fractions from pH gradient 5–7 (Fig. 4a); (c) fractions from pH gradient 6–6.5 (Fig. 4c). 0.1 ml of each fraction, adjusted to contain 5–15 μg of protein, were separated 2 h. "Du" represents BJP Du derived from DEAE-Sephadex (20 μg in 0.05 ml).

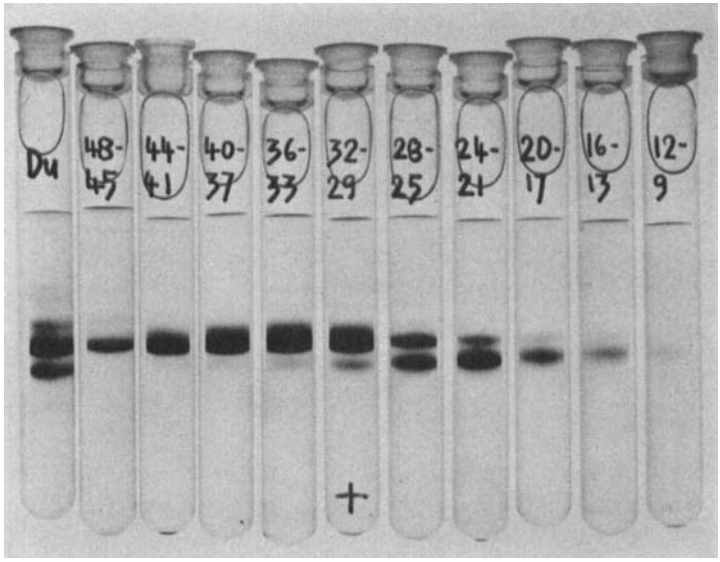

FIG. 3 (continued).

Overall protein recovery, calculated from O.D. readings of pooled aliquots of fractions 19–47 (Fig. 4a) after dialysis, was found to be 92%. Within the same pH range (Fig. 4b) O.D. measurement and evaporation to dryness of pooled fractions showed that carrier ampholytes are completely dialyzable.

Further resolution of the two main peaks in a still narrower pH gradient could be expected, but at present carrier ampholytes covering a range less than 2 pH units are not yet available commercially. For this reason the experiment shown in Fig. 4a was repeated by using the carrier ampholytes at a concentration of 3.3%. The separation could be reproduced accurately without influence from the elevated ampholyte concentration. Fractions 27–42 covering the range of pH 6.0–6.5 were rerun. For that purpose each of the 16 fractions was cut in two halves and the density of these 32 fractions adjusted with a concentrated sucrose solution and water to restore the original gradient as closely as possible. Calculation of sucrose concentrations was based on a calibration curve correlating local sucrose concentration in the original density gradient and fraction number. Final carrier-ampholyte concentration was estimated by calculation to be approximately 1%.

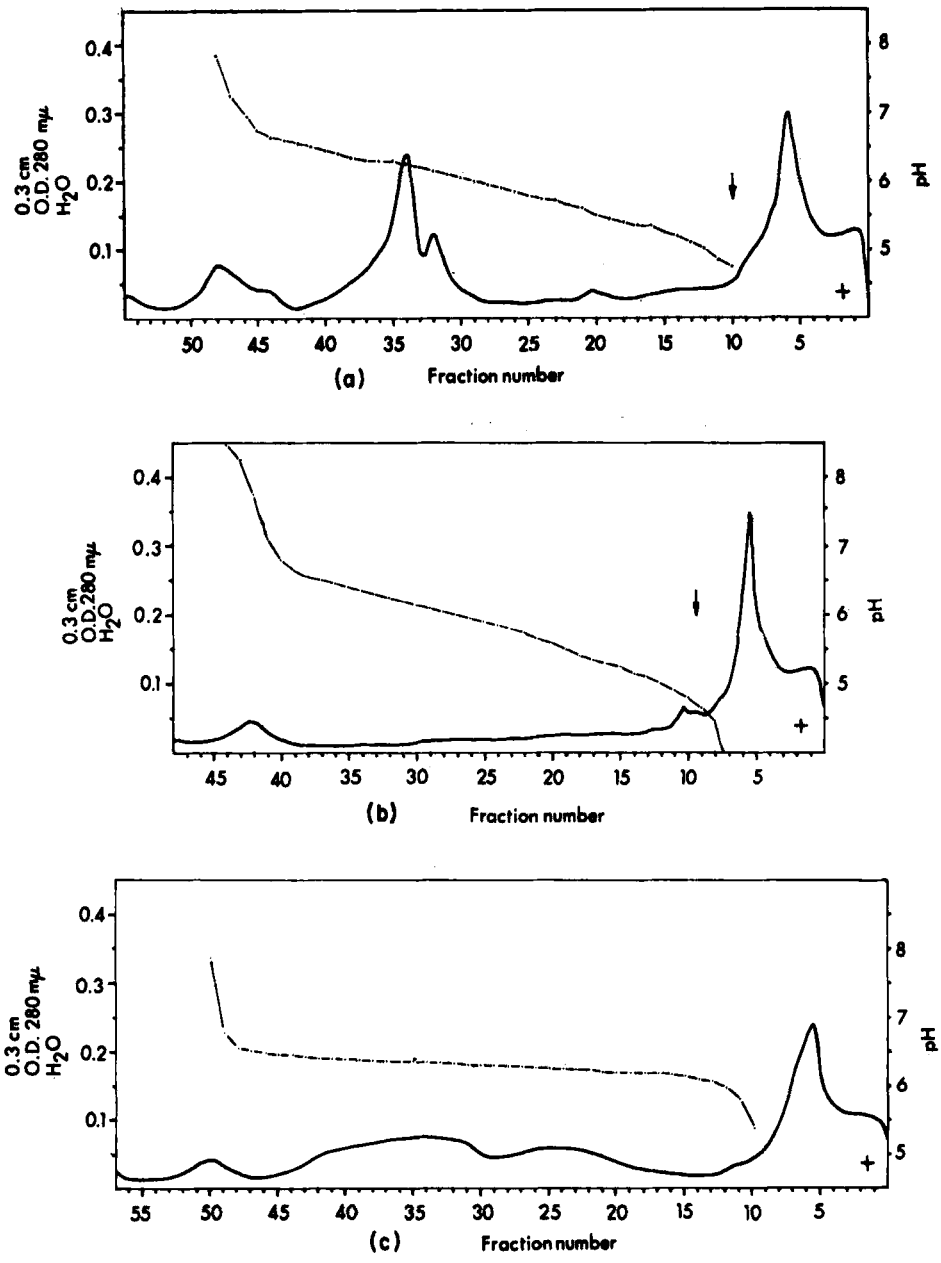

FIG. 4. Isoelectric separation experiments on BJP Du in narrow pH gradients: (a) pH gradient 5–7, 10 mg of protein; (b) pH gradient 5–7, no protein; (c) pH gradient 6–6.5. In each case the anode was at the bottom of the column. For further explanation see Fig. 2.

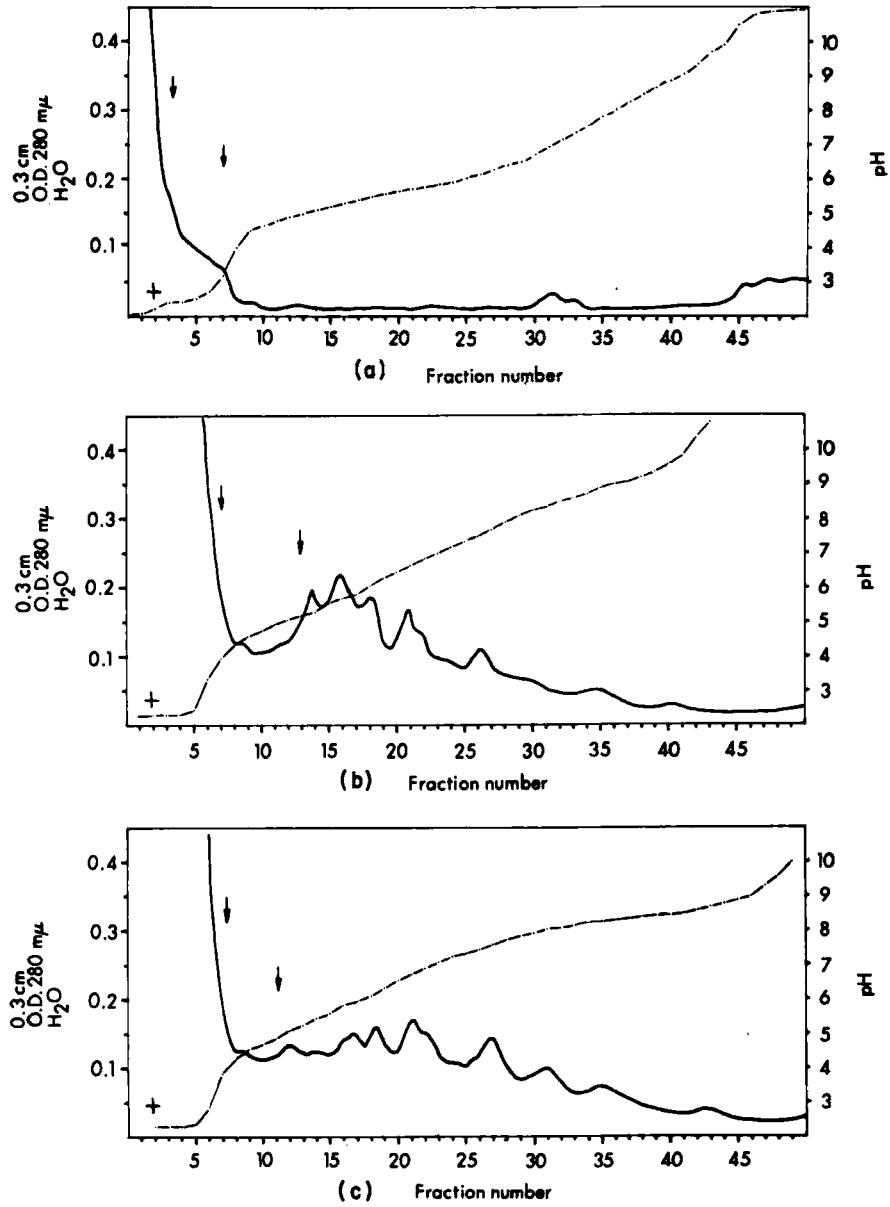

FIG. 5. Isoelectric experiments in 5 *M* urea on light chains of normal IgG in the pH gradient 3–10: (a) blank determination, no protein; (b) light chains prepared by oxidative sulfitolysis (10 mg); (c) light chains prepared by reduction/alkylation (10 mg). In each case the anode was at the bottom of the column. For further explanation see Fig. 2.

Voltage was set at 300 V at the beginning and increased to 700 V in the way mentioned before. The current was constant at 0.7 mA after 40 hr and fractionation was started after 68.5 hr. No precipitation was visible. The results of this separation are shown in Figs. 3c and 4c.

The mean values of the IEP of the two main components of BJP Du were estimated to be 6.35 and 6.20, respectively. The values could be reproduced in all the pH gradients mentioned wthin ±0.05 pH units under various conditions including urea.

Isoelectric Separation of BJP in the Presence of 5 M Urea

One of the limitations of the present method lies in the fact that many proteins have the minimum of solubility at the pH of their IEP. Furthermore some proteins are only sparingly soluble and/or may be denaturated if their solution is desalted. Under these circumstances the amount of material that can be separated must be substantially lowered. To avoid precipitation, urea may be used. Although none of the three BJP tested did precipitate during isoelectric separation, runs were made in 5 *M* urea for comparison. BJP Gr was separated within pH gradient 3–10 and BJP Du within pH gradient 5–7. In each experiment 10 mg of protein was used. Electrode positions, voltage, and currents were the same as in the runs without urea, but to avoid high initial currents urea of low conductivity had to be used. Precipitation was restricted to the electrode compartments, especially to the anode, which showed strong absorption in the O.D. tracings. Protein distribution, pH gradients, IEP, and disc-electrophoresis results were practically identical as compared to those of the runs without urea.

Isoelectric Separation of Light Chains of Normal, Pooled IgG

The solubility of the light chains prepared under the methods described was low in aqueous solutions devoid of urea. Separations were therefore carried out in the presence of 5 *M* urea. The pH gradient from 3–10 was chosen on account of the well-known heterogeneity of light chains in gel electrophoresis. In each experiment 10 mg of protein was used. Because urea of low conductivity was not at hand at the time, voltage was gradually increased from 120 V to a constant value of 300 V within the first 2 hr. The current decreased to a constant value of 0.7–0.8 mA after 35–40 hr and fractionation was started after 47–50 hr. No precipitation occurred outside the electrode compartments in the blind run (Fig. 5a). During the separation of light chains (Figs. 5b,c) prepared by oxidative sulfitolysis (L_{os}) and reduction/

alkylation ($L_{r/a}$) opalescent bands in the fractions immediately above the lower electrode could be observed after the first 24 hr. The bands remained stationary throughout the separation and were more pronounced with L_{os}. Most of the proteins were focused in the gradient between pH 5.0–8.0 (L_{os}) and pH 5.3–8.2 ($L_{r/a}$). Disc electrophoresis (Figs. 6a,b) showed partial resolution.

DISCUSSION

Limiting factors in preparative isoelectric separation are the isoelectric solubility and the stability at low ionic strength of the components under investigation. These experiments have shown that the solubility of the three BJP as well as light chains of normal IgG was such that at least 5–10 mg of these proteins could be handled in a 110-ml column. Although usually some precipitation could be observed, it was located or primarily originated in the electrode compartments. Whatever the nature of these precipitates [Earland and Ramsden (*12*)], the fact should be taken into consideration that all ionic constituents that do not lose their charge in the range of the pH gradient migrate toward the electrodes. However, as judged from their migration in disc electrophoresis, proteins were not denaturated and recoveries were close to 100%. In the case of BJP Fi precipitation disturbed separation, but by reversal of electrode polarity this difficulty could be overcome.

As already mentioned, solubility might be increased by adding urea to the gradient. Though this effect could not be demonstrated with the soluble BJP, it was rather surprising to note the close agreement of results obtained in the presence and absence of urea. In the case of light chains, urea was indispensable because of the low solubility of the protein, which is a consequence of the method of preparation.

Other means to improve column capacity can be narrower pH gradients and higher carrier-ampholyte concentration. On the other hand, isoelectric separation of small amounts of material in the analytical range might provide useful information concerning IEP and purity. Finally, low isoelectric solubility might be used for the specific removal of single components from an otherwise soluble mixture.

Heterogeneity of all three BJP was indicated by the experiments carried out in the pH range 3–10, but disc electrophoresis showed substantial overlapping of the resolved proteins. It could be demonstrated that the resolving power improved with decreasing pH range. Because the experiments in the pH gradients 5–7 and 6–6.5 were both

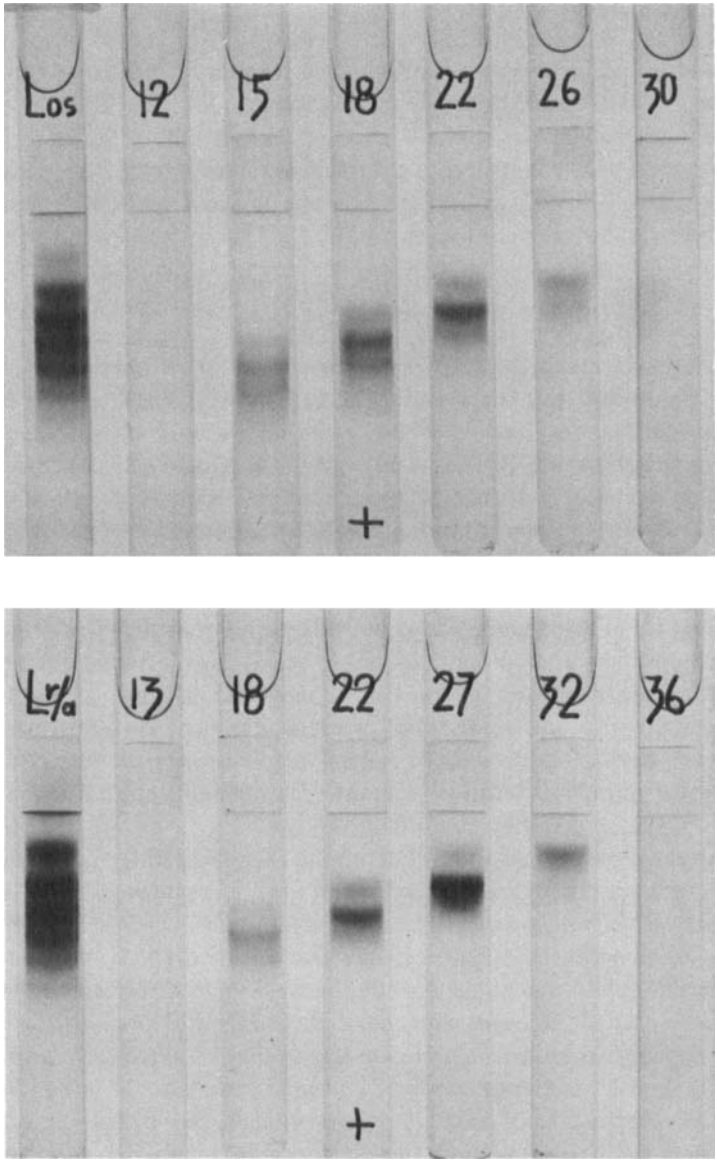

FIG. 6. Disc electrophoresis in 10 *M* urea of light chains after isoelectric separation: (a) light chains prepared by oxidative sulfitolysis (compare Fig. 5b); (b) light chains prepared by reduction/alkylation (compare Fig. 5c). 0.1 ml of each fraction containing 5–15 μg of protein were separated during 2 h at 3–4 mA/tube and 200 V. L_{os} and $L_{r/a}$ are light chains, starting material, 30 μg in 0.1 ml.

carried out at a final potential of 700 V, zone width in the shallower pH gradient was larger. According to Svensson (*2*) and Vesterberg and Svensson (*5*) further separation can be expected at higher field strength.

The IEP of the two main components of BJP Du could be reproduced under a variety of conditions within the accuracy of the pH meter. The difference in the IEP of these two components was 0.15 ± 0.05 pH units. The separation was not affected by increasing the carrier ampholyte concentration from 1 to 3.3%.

In the blank experiments without protein, uv absorption was not uniform over the entire pH range. This is a reflection of the indispensable molecular heterogeneity of the carrier ampholytes.

The isoelectric separation of light chains, realized in pH gradients 3–10, showed partial resolution of the many bands well-known from gel electrophoresis. Compared with $L_{r/a}$ the zones of L_{os} were slightly shifted toward the acidic side of the pH gradient. This can be explained by the extra charge introduced ($S\text{-}SO_3^-$) during their preparation. Further separation of light chains, perhaps the isolation of single bands, should be possible by using much narrower pH gradients at a high field strength.

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