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METHOD FOR IMPROVED PROTEIN SEPARATION IN HUMAN AND ANIMAL SERA

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SUMMARY

A method of improved protein separation in human and animal sera on columns of diethylaminoethyl cellulose is described. The improvement in protein separation is achieved by employing suitably modified elution gradients, the construction of which is described. A comparison with other work shows that improved resolution has been achieved over shorter elution times.

INTRODUCTION

Ion-exchange chromatography is a valuable tool for the separation and isolation of proteins and other biological macromolecules and the technique can be applied in many biochemical fields[1–6].

The most commonly used cellulosic ion exchangers are DEAE-cellulose, an anion exchanger, and CM-cellulose, a cation exchanger.

Protein elution on DEAE-cellulose can be achieved in a number of ways[5], but the most popular method is to use a varying salt (commonly NaCl) concentration superimposed on a buffer of fixed composition. In most previous work a linear salt gradient has been used since this type of gradient is most easily set up. However, work has been done employing a number of non-linear salt gradients[7] and this work shows that, by suitable modification of the gradient, sufficient improvement in protein resolution can be obtained as to facilitate the isolation of the constituent proteins in animal and human serum.

When a protein molecule is passed through a column of DEAE-cellulose, it may become adsorbed onto the surface of the cellulose by ionic attraction. Some proteins, notably γ-globulin, are not adsorbed onto the cellulose and merely pass straight through the column.

Each adsorbed protein molecule is ionically bound to the DEAE-cellulose, the binding strength varying from protein to protein according to the relative number, arrangement and ionic behaviour of its amino acid groupings, and, in order to remove the protein from the cellulose, the bond between the two has to be broken by replacing the amino acid groups of the protein by the chloride functional group on the substituted cellulose. The concentration of chloride ion needed to do this will thus vary

according to the protein, *i.e.*, each protein will be removed from the cellulose by a specific NaCl concentration.

If two proteins adsorbed onto the cellulose are removed by only slightly differing NaCl concentrations, and a linear NaCl gradient is employed, by the time the first removed reaches the detector the concentration of NaCl in the column will have risen enough to remove the second protein from the cellulose and carry it to the detector. This will result in a poor separation of the two proteins. This work shows that, if the NaCl concentration is held constant until the first protein has passed through the detector, improvement in resolution is obtained.

METHODS AND PROCEDURES

The system employed is shown in Fig. 1. The cellulose used in the experiments was DEAE-cellulose 200–300 mesh (55–75 μm nominal) obtained from Viscose, Swansea, Great Britain. The columns used in this work were standard 30 cm × 1.5 cm I.D. cylindrical glass columns obtained from Viscose. The bovine serum was obtained from Koch-Light Labs., Colnbrook, Great Britain.

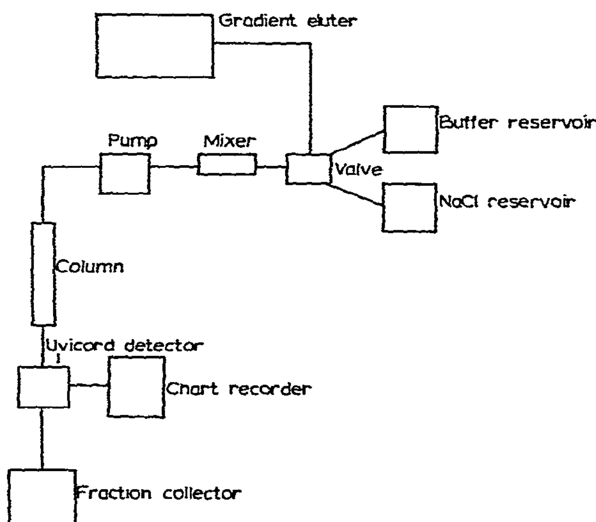

Fig. 1. Schematic diagram of apparatus.

Isolation of blood plasma

Human blood was obtained from a blood bank. The blood was centrifuged at 14,000 *g* and the yellow plasma that separated from the red blood fraction was drawn off. The plasma was stored in a refrigerator at 4°.

Precycling of medium

The cellulose medium was obtained as a wet cake. To ensure complete swelling, and further purification, the medium was precycled by soaking in 15 volumes of 0.5 *M* HCl for 30 min, followed by washing with deionized water and soaking in 0.5 *M* NaOH

for 30 min. The medium was then washed thoroughly with deionized water until the washings were neutral. The medium was then equilibrated with the starting buffer by immersing sufficient medium for a column in 8 l of buffer (in this case, 0.01 *M* Tris·HCl, pH 7.5) for 30 min, stirring at intervals, decanting off most of the buffer and adding a further 8 l of fresh buffer. After a further 30 min of stirring, the buffer was decanted and the medium filtered to a wet cake.

Column packing

The equilibrated medium was stirred into 1–2 volumes of starting buffer and placed under a gentle vacuum to remove air bubbles. The slurry was then poured into the column via an extension tube about 130 cm in length keeping the column exit tap closed until about 1 cm of medium had settled at the bottom of the column. The tap was opened to expedite column packing, making sure that the top of the column did not run dry.

After the column had settled, at least one column-volume of buffer was passed through the media before applying the sample.

Sample preparation

Bovine serum solution was made up by allowing 0.3 g of serum to dissolve in 10 ml of distilled water in a refrigerator. The solution was then dialysed in 5 l of 0.01 *M* Tris·HCl buffer pH 7.5 for 24 h in the refrigerator.

Undiluted human blood plasma was dialysed in the same way.

Sample application to column

Five millilitres of dialysed sample were applied evenly to the column when the buffer front was just disappearing into the surface of the medium. The protein was allowed to sink into the medium and, when the liquid front was just vanishing into the medium, the column was "topped up" with buffer and the Gradient Elutor switched on to begin elution.

Gradient construction

The desired gradient for improved resolution of serum proteins was obtained as follows.

An initial test chromatogram was obtained employing a linear 0–0.4 *M* NaCl gradient under identical conditions of flow-rate and time as were to be used for non-linear gradients. Such a chromatogram contained broad, poorly resolved protein peaks (Fig. 2, solid line). It was possible to superimpose the gradient employed onto the chromatogram, as shown in Fig. 2 (solid line), and by interpolation from the chromatogram the NaCl concentration at which each peak is removed from the column could be found. The gradient was then modified into a series of steps (Fig. 2, broken line), the NaCl concentration at each step being that required to remove that particular protein from the column, and the length of each step being sufficient to ensure the complete elution of each protein before the NaCl concentration was increased to the next step level. In order that sudden changes in NaCl concentration did not occur, the gradient was cut at an incline between each step, as shown in Fig. 2 (broken line).

The chromatogram produced by this stepped gradient is shown in Fig. 2

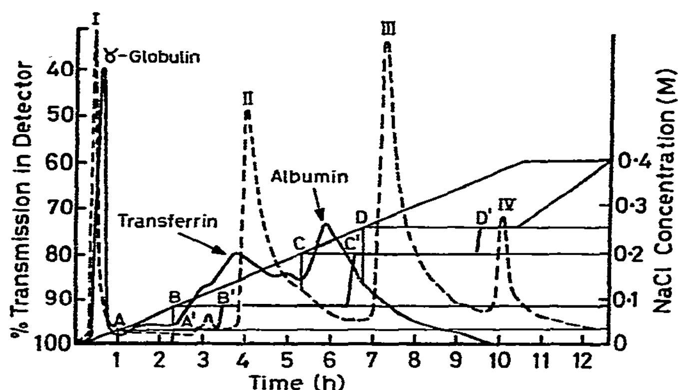

Fig. 2. Separation of bovine serum proteins using a linear NaCl gradient ABCD (———) and using stepped gradient A'B'C'D' (— — —), derived from ABCD.

(broken line). The improvement in protein resolution and peak sharpness is evident. Further improvement was achieved by successive modification of the gradient using the above method.

In some cases where further resolution of proteins is desirable it is possible to incorporate an LKB Sensor in the system. This is an electronic switch that holds the gradient at a fixed composition when the detector response reaches a present level. The benefit from using the Level Sensor is that the protein peaks will be more spaced out, although the total elution time will be greater.

RESULTS AND DISCUSSION

Bovine serum

Fig. 2 (solid line) shows a chromatogram of bovine serum proteins using a linear 0–0.4 *M* NaCl gradient. This chromatogram is essentially identical to that obtained under similar conditions by the serum manufacturers[8]. The initial improvement, using the stepped gradient, in protein separation is shown by the broken line. The solid line shows a sharp initial γ-globulin peak and two broad bands containing transferrin and albumin as indicated in the diagram. The broken line shows four large protein bands I–IV. No change is observed in the γ-globulin peak (I) since this protein is not adsorbed onto the cellulose. Although having sharp maxima, bands II and III show significant tailing. This is indicative of the presence of further proteins. In order to resolve these proteins, intermediate steps between A' and B' and B' and C' were introduced into the gradient and the final resulting chromatogram is shown in Fig. 3 (broken line).

Fig. 3 (solid line), which is the same as Fig. 2 (solid line), is included for comparison. The improvement in protein separation is evident and little or no resolution is lost by doubling the flow-rate with a halving of the elution time, as shown by Fig. 4 (broken line).

This may be of importance in, for example, the extraction of proteins from effluent streams since high flow-rates and short elution times would be necessary in such systems.

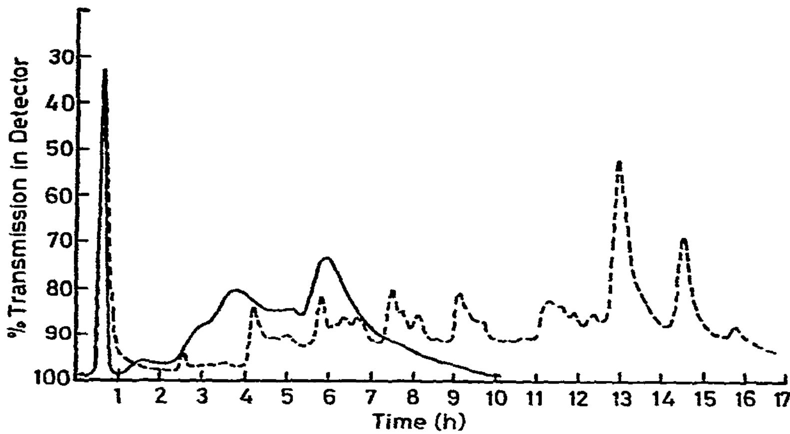

Fig. 3. Separation of bovine serum proteins using a linear 0–0.4 M NaCl gradient at a flow-rate of 40 ml/h (———) and the same separation using final stepped gradient (– – –).

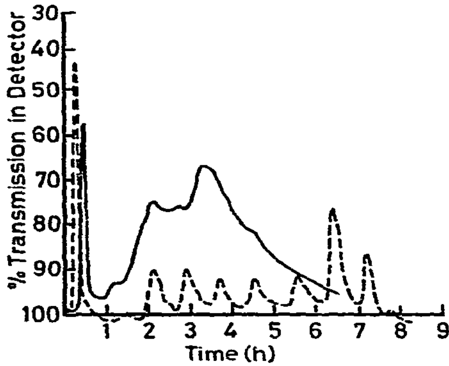

Fig. 4. The same separations as in Fig. 3 at double the flow-rate (80 ml/h) over half the elution time. ———, Separation with the linear gradient; – – –, separation with the stepped gradient.

Human plasma

Fig. 5 (solid line) shows a chromatogram of human plasma proteins obtained by using a linear 0–0.4 M NaCl gradient.

Fig. 5 (broken line) shows a chromatogram obtained under identical conditions using the final stepped gradient for human plasma derived originally from Fig. 5 (solid line). The improvement in protein resolution is again evident, there being over a dozen separate sharp protein peaks.

Fig. 6 (broken line) again shows that little resolution is lost by doubling the flow-rate and halving the elution time.

Fig. 7 shows the chromatogram obtained by incorporating the Level Sensor in the system.

A comparison with Fig. 5 (broken line) shows that the protein peaks are much more spaced out when the Level Sensor is employed. This would be of value in the fractionation and isolation of proteins from the protein peaks. Under conditions where

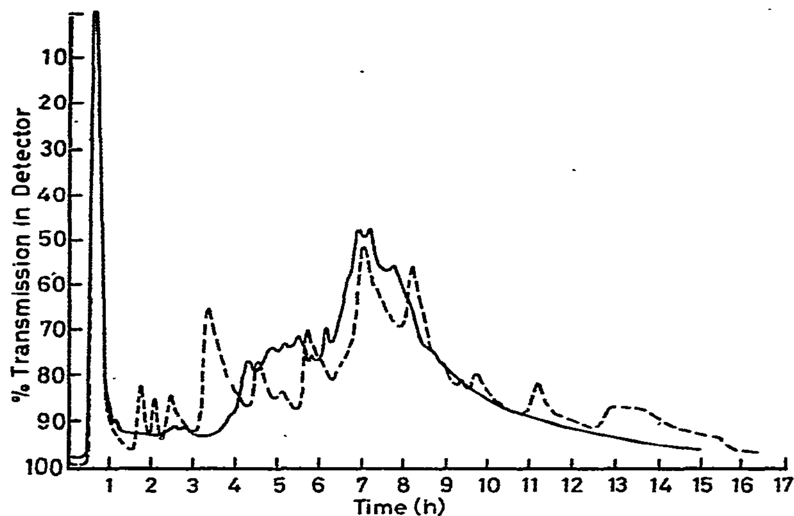

Fig. 5. Separation of human plasma proteins on a linear 0–0.4 *M* NaCl gradient at a flow-rate of 40 ml/h over an elution time of 16 h (———) and the same separation using final stepped gradient (– – –).

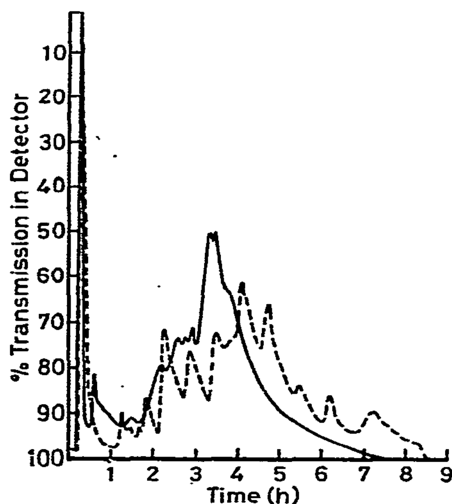

Fig. 6. The same separations as in Fig. 5 at double the flow-rate (80 ml/h) over half the elution time. ——, Separation with the linear gradient; – – –, separation with the stepped gradient.

the dead volume of a system such as that employed in this work is minimal (less than about 10 ml) the Level Sensor would replace the need for cutting steps in the gradient chartpaper since it performs the same function in effect by holding the gradient at a constant composition during the elution of a protein. However, in the system employed above the dead volume precluded the use of the Level Sensor as the sole method for holding the gradient at a constant composition.

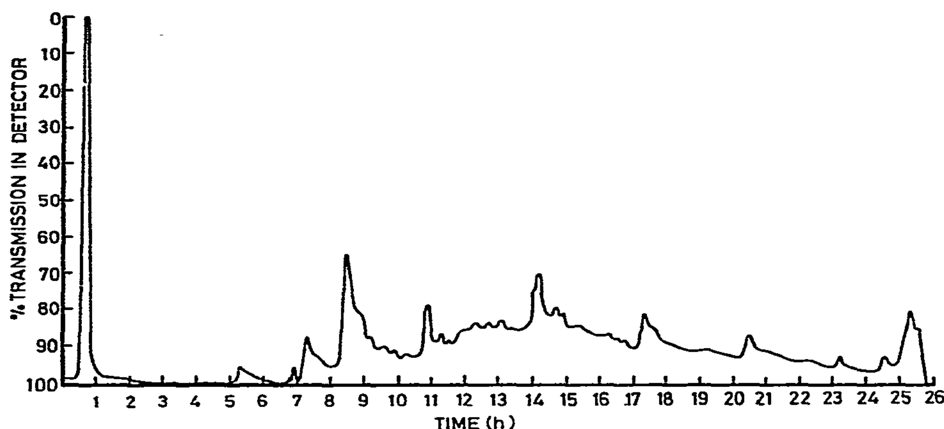

Fig. 7. Separation of human plasma proteins under the conditions as for Fig. 5 incorporating the LKB Level Sensor in the system.

The technique has shown that excellent protein resolution can be obtained in a time much less than those used in other systems[7]. It is possible to extend the use of the technique to the fractionation and identification of each protein peak. As the chromatograms are accurately reproducible the technique can be used as an analytical tool for the detection of certain proteins. An important application of the technique may be in detecting blood disorders by studying the protein deficiencies or abnormal ratios of proteins in the blood.

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