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SEPARATION AND CHARACTERISATION OF GLYCOPROTEINS FROM NORMAL, PREGNANCY, AND ACUTE INFLAMMATORY SERA

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SUMMARY

A system of multiple chromatography combining anion-exchange, gel filtration, and affinity chromatography has been devised to separate several acute phase proteins from serum, including α_1 acid glycoprotein, transferrin, α_1-antichymotrypsin, α_1-antitrypsin and haemopexin. Only 5–15 ml of blood is required to provide milligram quantities of the purified proteins for further biochemical analysis. The system has been applied in a study of pregnancy and some diseases in order to investigate the changes in the serum protein profile and in individual proteins.

INTRODUCTION

There is an extensive literature on the chromatographic isolation of many plasma proteins by gel filtration and ion exchange[1]. The majority of these methods require large volumes of plasma as the starting material. More recently, a range of affinity chromatographic systems have been used to isolate minor proteins. Electrophoretic techniques have shown evidence of microheterogeneity of many plasma proteins of genetic origin as well as minor changes, such as the loss of sialic acid residues and variation in glycan branching that occur in pregnancy and disease[2]. In order to study the relationship of structural change and protein function in disease, it is necessary to isolate the proteins and work within the restraints of a volume of blood compatible with a single donor sample, often from an ill patient.

Our aim was to develop a rapid separation system that can handle 10–20 ml of blood to isolate several key glycoproteins. Serum is a highly complex mixture of proteins with a wide variety of concentrations, *e.g.* albumin (Alb), 40 g/l; IgG, 12 g/l; α_1-antichymotrypsin (ACT), 0.5 g/l. The proteins of interest are in the 40–100 kilodalton range, several of them have closely related p*I* values, and they often exhibit charge heterogeneity. Our approach was the development of a chromatographic sequence that would attain optimum separations. A Fast Protein Liquid Chromatography System™ (FPLC) which provides high resolution and automation, was the basic instrumentation; this was used in association with gel-filtration, anion-exchange, and affinity chromatography to isolate several proteins that may be modified in disease, *e.g.* ACT, α_1-acid glycoprotein (AGP), vitamin D binding globulin (Gc), and transferrin (Tf), α_1-antiprotease (AT), and antithrombin III (ATIII). We pre-

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viously reported the initial development of such a system, which has now been modified and applied to study sera in pregnancy and in some diseases[3].

EXPERIMENTAL

Materials

Serum was obtained from patients or healthy volunteers and stored at 4°C. Chromatography was carried out using a Pharmacia FPLC™ system with a LCC-500 controller, two P-500 pumps, and a UV-M monitor with a 5-mm flow cell. All columns (Mono Q HR 5/5 and HR 16/10) and gels (Sephacryl 200, Blue Sepharose CL 6B, and Heparin Sepharose) were obtained from Pharmacia (Uppsala, Sweden). All chemicals were obtained from BDH (Poole, U.K.) or Sigma (Poole, U.K.). All protein solutions were concentrated using an Amicon ultrafiltration cell (50 or 250 ml) with a YM 5 membrane (Amicon, Gloucs., U.K.). The monospecific rabbit antisera were obtained from Dako (High Wycombe, U.K.) and Behringwerke (Marburg, F.R.G.).

Electrophoretic methods

The position of individual proteins eluted from the columns was determined by rocket electroimmunodiffusion with specific antisera[4]. The purity and composition of fractions was analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) using the PhastSystem™ (Pharmacia). The PhastSystem is a fully automated rapid electrophoresis separation and staining system. The gels are 50 × 43 × 0.45 mm and use agarose buffer strips. Samples (1 μl) of the fractions were loaded onto a 10–15% gradient SDS-PAGE system and electrophoresed for 60 Vh, at 250 V, 3.0 W, and 15°C. They were then stained with Phastgel Blue®, the whole process taking 90 min. Lectin crossed affinoimmunoelectrophoresis using concanavalin A was carried out as described by Wells *et al.*[5]. The concentration of individual protein was determined by a modification of the radial immunodiffusion method of Mancini *et al.*[6].

RESULTS AND DISCUSSION

Our approach to the separation was to remove first Alb and IgG by affinity chromatography, as these represent more than half the total proteins in serum and limit the loading of the various chromatographic columns. The second step was to divide the residual serum into two broad groups containing the higher- and lower-molecular-weight proteins. The proteins of interest were in the low-molecular-weight fraction (LMW), 40–100 kilodalton. Because the lipids present in serum have a detrimental effect on the life of the columns, they were removed by hexane extraction. Briefly, an equal volume of hexane was added to the serum and the mixture Vortex-mixed and then centrifuged for 15 min at 1000 *g*. The bottom layer was then applied to a column of Blue Sepharose CL-6B in series with a Protein A Sepharose column in order to remove the Alb and IgG, respectively (Fig. 1). These proteins account for 80% of the total protein content of serum. Several plasma proteins can bind to Blue Sepharose and are eluted by differing sodium chloride concentrations[7]. Albumin binds the strongest, being eluted at >0.4 *M* sodium chloride. For this reason, the

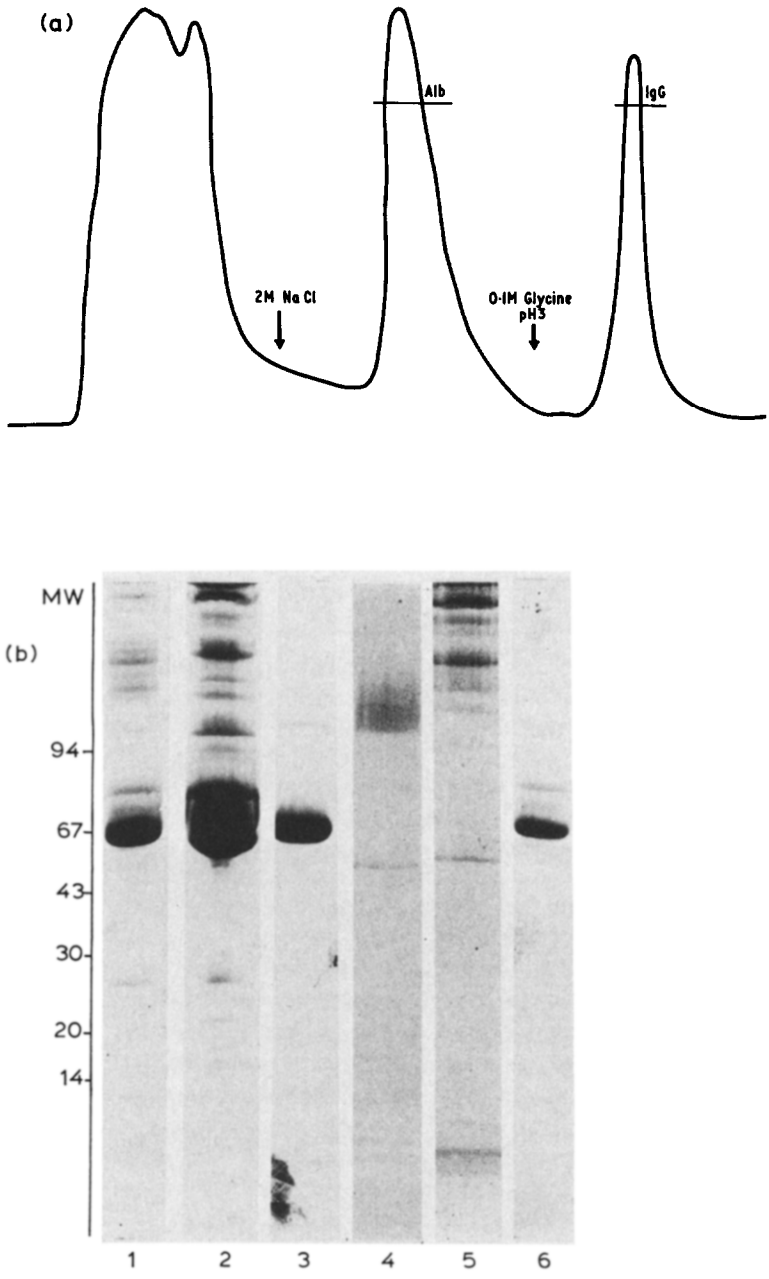

Fig. 1. Removal of albumin and IgG. (a) A 15-ml serum sample was loaded onto a Blue Sepharose CL-6B column (10 × 1.6 cm) in series with a Protein A Sepharose column (11 × 1 cm) equilibrated with 50 m*M* sodium phosphate, 0.4 *M* sodium chloride, pH 7.2. Flow-rate: 1 ml/min, fraction size: 1 ml. The Alb was eluted with 2 *M* sodium chloride in the above buffer; IgG was eluted with 0.1 *M* glycine, pH 3. (b) The three eluents were pooled and 1-μl samples of each fraction analysed by SDS-PAGE as shown; Tracks: (1) Serum, (2) Blue Sepharose–Protein A non-bound, (3) Alb, (4) IgG.

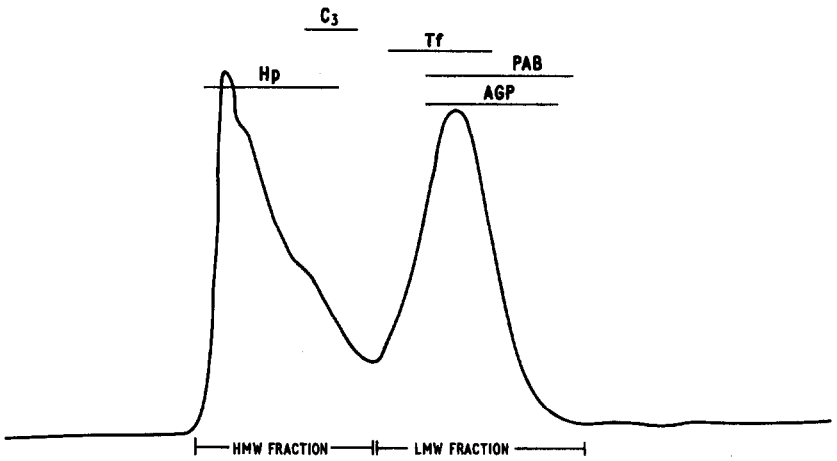

Fig. 2. Preparation of low-molecular-weight (LMW) fraction. The unbound proteins from the Blue Sepharose column were loaded onto a Sephacryl 200 gel filtration column (75 × 2.6 cm) equilibrated with 50 m*M* sodium potassium phosphate, 0.15 *M* sodium chloride, pH 7.2 buffer. Flow-rate: 0.3 ml/min, fraction size: 5 ml, overnight at 4°C. SDS-PAGE (see Fig. 1b) shows the composition of the HMW fraction (5), and the LMW fraction (6).

serum was loaded in 0.4 *M* sodium chloride, so that only Alb was bound, whilst the other proteins passed through the column. This salt concentration still allowed IgG (subclasses 1, 2 and 4) to bind to the Protein A Sepharose.

The proteins not bound to the Blue Sepharose and Protein A columns were concentrated to 5 ml and subjected to gel filtration on Sephacryl 200 gel, as shown in Fig. 2. The first peak contained high-molecular-weight proteins (> 100 kilodalton),

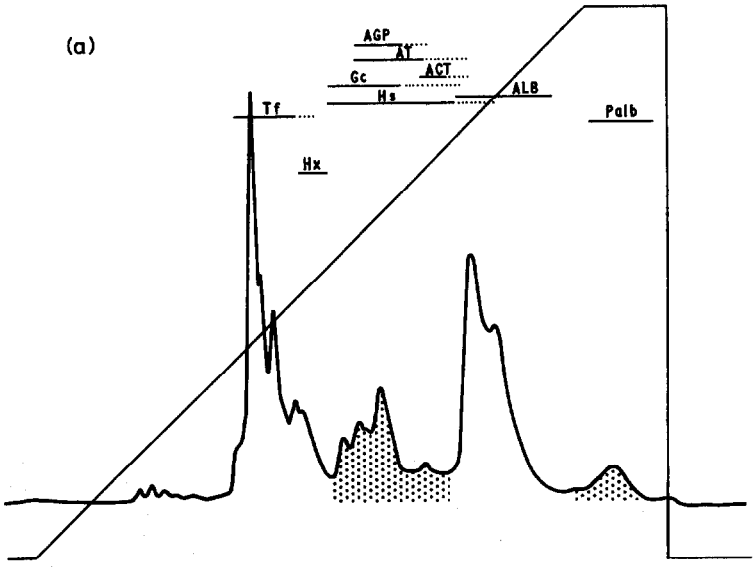

Fig. 3.

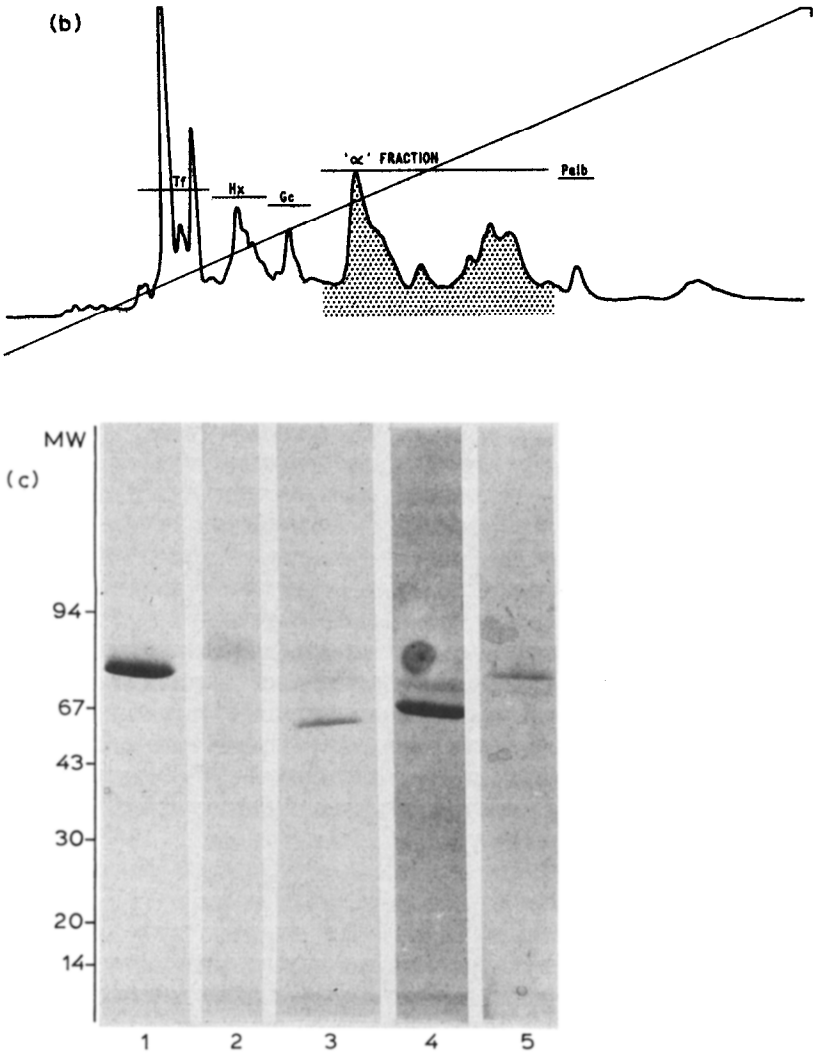

Fig. 3. Anion exchange chromatography. (a) The LMW fraction was applied to a Mono Q HR16/10 (10 × 1.6 cm) anion exchanger column equilibrated with buffer A (20 m*M* bis Tris propane, pH 7.5). It was eluted with a 400-ml, 0–100% gradient of buffer B (20 m*M* bis Tris propane, 0.35 *M* sodium chloride, pH 9.5. Flow-rate: 10 ml/min, fraction size 10 ml. (b) The composition of buffer B was altered to 20 m*M* bis Tris propane, 0.7 *M* sodium acetate, pH 9.5 and the gradient volume increased to 1000 ml. The increased resolution allowing the isolation of several protein is shown. (c) SDS-PAGE of the isolated proteins; Tracks (1) Tf, (2) Hx, (3) Gc, (4) "α", (5) prealbumin (Palb).

whilst the second peak contained proteins of 40–100 kilodalton. Caeruloplasmin was present in the trailing edge of the first peak. This fact must be taken into account when high yields of this protein are required. The second peak fraction, the LMW fraction, was pooled and concentrated to 10 ml prior to anion-exchange chromatography.

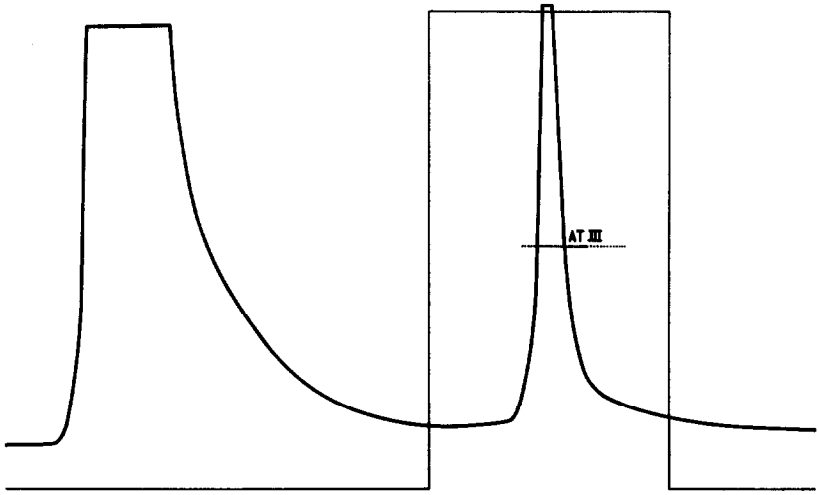

Fig. 4. Separation of ATIII. The "α" fraction was applied to a Heparin-Sepharose column (10 × 1 cm) in 10 m*M* sodium phosphate, pH 7.2. Flow-rate: 0.5 ml/min, fraction size: 1 ml. The ATIII was eluted with a 0–100% gradient of 10 m*M* sodium phosphate, 1.5 *M* sodium chloride, pH 7.2.

The LMW fraction was then separated on a Mono Q anion exchanger. Initially, a bis Tris propane buffer with a sodium chloride gradient, developed for separating urinary proteins, was used with the Mono Q HR5/5 column[8]. This resulted in the LMW proteins separating into three main groups (Fig. 3a). By altering the cation from chloride to acetate the resolution was slightly improved. However, by optimising the gradient volume the resolution was improved still further. These final con-

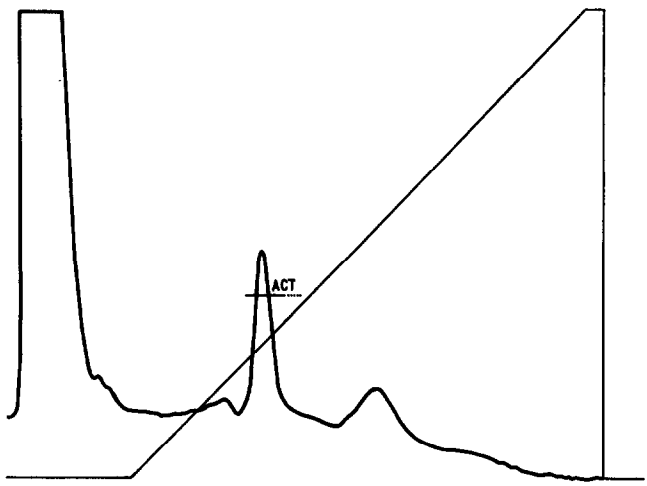

Fig. 5. Separation of ACT. The unbound void from the Heparin-Sepharose column was applied to a Blue Sepharose column (16/10), in 10 m*M* sodium phosphate, pH 7.2. Flow-rate: 1 ml/min, fraction size: 1 ml. The bound ACT was eluted with a 0–1.5 *M* sodium chloride gradient in the same buffer.

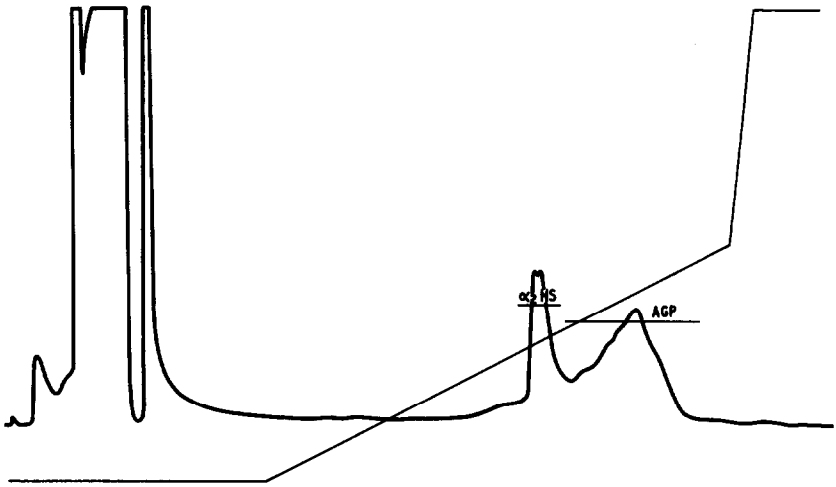

Fig. 6. Preparation of AGP. The Blue Sepharose unbound fraction was applied to a Mono Q HR5/5 column equilibrated in 20 m*M* EPPS buffer, pH 3.8. Flow-rate: 1 ml/min, fraction size: 1 ml. AGP bound to the column and was eluted with a 20 ml, 0–0.35 *M* sodium chloride gradient.

ditions (Fig. 3b) were then used and the system was scaled up by a factor of 20 to a Mono Q HR16/10 column. This resolved Tf, haemopexin (Hx), Gc, and Palb. The remaining fractions were pooled, as shown, and concentrated. They contained the "α"-mobility proteins, *e.g.* ACT, AGP, ATIII (Fig. 3c). Due to their microhetero-

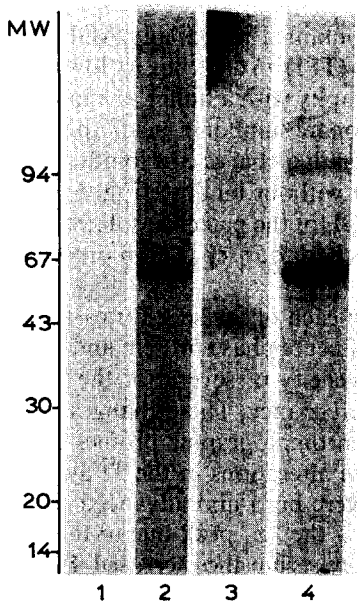

Fig. 7. SDS-PAGE of "α" fractions. This shows the various stages of the "α" fraction separation and the purified proteins obtained; (1) ATIII, (2) ACT, (3) AGP, (4) AT.

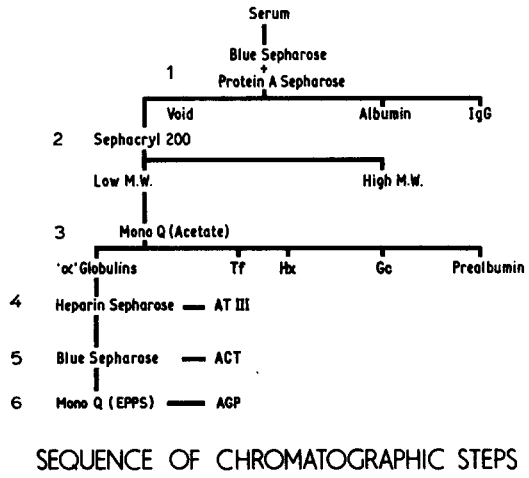

Fig. 8. Sequence of chromatographic steps showing the isolation of various serum proteins.

geneity, it was not possible to separate them by chromatofocusing. At this stage, affinity chromatography was used to provide a third "dimension" for the separation.

The ATIII was the first protein to be isolated from the "α" fraction by a Heparin-Sepharose affinity step[9] (Fig. 4). This allowed Blue Sepharose CL-6B to be used to isolate ACT[10]; because ATIII also binds to Blue Sepharose and coelutes with the ACT, it is necessary to remove it first. Salt was not added to the equilibration buffer for the Blue Sepharose in order to enhance the binding of ACT as shown in Fig. 5.

The void fraction from the Blue Sepharose contained principally AGP, AT, and a very low amount of thiol proteinase inhibitor (TPI). AGP with its low p*I* of 2.7 is easily separated by anion-exchange chromatography with a buffer at a low pH. EPPS buffer [N-(2-hydroxyethyl)piperazine-N'-3-propanesulphonic acid] (pH 3.8) was used[11]. At this pH, only AGP has a strong negative charge and binds to the column, the other proteins pass through the column without binding (Fig. 6). This unbound fraction is AT of 90% purity. The SDS-PAGE of the proteins isolated from the "α" fraction are shown in Fig. 7. The final sequence for our separation procedure is shown in the flow chart in Fig. 8.

The concentration of the individual proteins in each of the sera varies greatly depending on the patient's clinical status. The level of acute phase protein and transferrin being particularly labile and the microheterogeneity of several of the glycoproteins being considerably modified in disease and pregnancy. These changes in the LMW fractions of serum protein seen in Mono Q chromatographic profiles at the third stage of separation are illustrated in Fig. 9. The alterations in the Tf and "α" protein concentrations are the most apparent. In severe burn and advanced cancer sera the Tf peaks are very small, the major peak being the "α" peak due to the high concentrations of ACT, AT anmd AGP. In pregnancy serum the increased Tf level results in two peaks of almost equal height without a equivalent increase in "α" proteins. By contrast renal failure exhibits two principal peaks, Tf and "α" peaks resulting from the high AGP values.

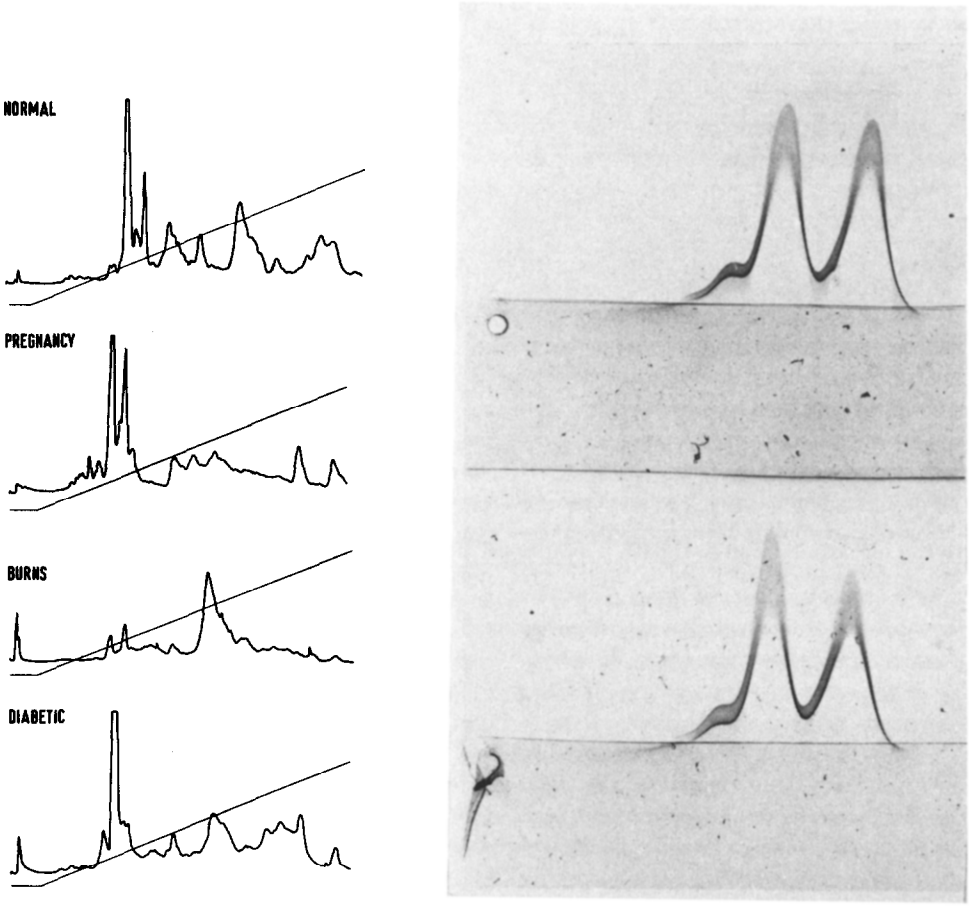

Fig. 9. Separation of the LMW fractions on Mono Q demonstrating the altered profiles in the disease sera compared to normal serum.

Fig. 10. Lectin crossed affinoimmunoelectrophoresis of AGP with concanavalin A in the 1st dimension gel. (top) Normal serum AGP, (bottom) purified AGP from sample.

As the purified serum glycoproteins are to be used in further biochemical analyses, including carbohydrate analysis, it is important that the purification methods used have not resulted in any alterations of the protein structure. The steps involved in this separation sequence were designed to minimise any such effects by avoiding buffers with extremes of pH. Lectin crossed affinoimmuelectrophoresis using concanavalin A in the first dimension gel, showed that the purified AGP retained the original serum AGP pattern, indicating no major change in the proportion of bi- and tri-antennary glycans (Fig. 10), although it would not identify minor alterations in the sialylation.

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