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ISOLATION OF IMMUNOGLOBULIN M FROM HUMAN SERUM BY GEL CHROMATOGRAPHY AND ELECTROCHROMATOGRAPHY ON A COLUMN OF SEPHADEX G-200

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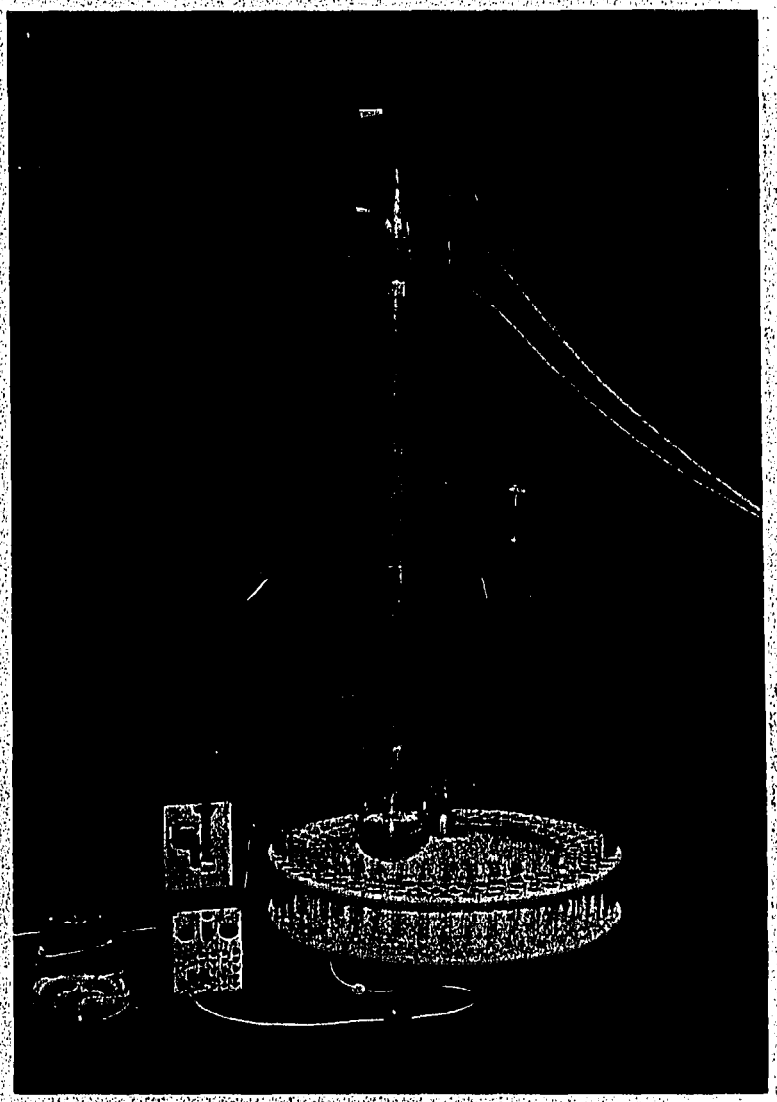
SUMMARY

Gel chromatography of human serum on a column of Sephadex G-200 gave the three standard fractions. From the first of these (19S), containing IgM, α_2-macroglobulin and β_1-lipoprotein, IgM has been isolated by gel chromatography combined with simultaneous electrophoresis. The electrochromatographic separation was carried out on a column of original construction, described in detail.

INTRODUCTION

The gel chromatography of serum on a column of Sephadex, described by FLODIN AND KILLANDER[1,2] in 1962 and treated in a number of subsequent communications (*e.g.*, refs. 3–8), is known to yield three fractions, the first of which contains IgM, α_2-macroglobulin and β_1-lipoprotein as the major components. Owing to the sedimentation velocity of IgM as the most important constituent, this fraction and its elution peak are generally designated 19S. Separation of IgM from the other two components of fraction 19S is the subject of the present paper.

Comparison of the elution peaks of IgM alone (immunodiffusion plate assays) and of the whole fraction 19S with respect to their positions on the axis of abscissae has revealed that IgM advances faster in the conventional gel chromatography than either or at least one of the other two proteins. However, the difference is so small that the three components overlap throughout the elution peak 19S (immunoelectrophoretic analysis). As they also differ in electrophoretic mobility, we exposed the isolated fraction 19S to the combined effect of gel chromatography and electrophoresis. Column electrochromatography has been described before[9–12], but isolation of IgM by this method has not previously been attempted. As the electrochromatographic column constructed for this purpose is rather different in design from those hitherto proposed, it is described in detail.



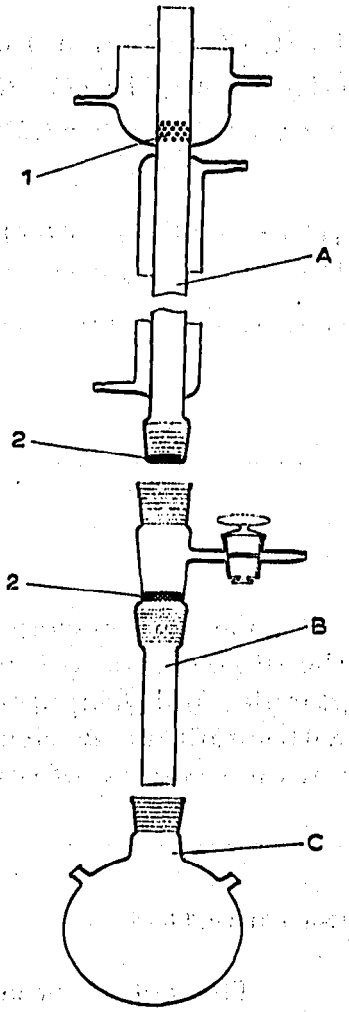


Fig. 1. Photograph of the apparatus.

Fig. 2. Parts of the column. 1 = ringlet of minute holes; 2 = sintered glass. Parts A, B and C are described in the text.

EXPERIMENTAL

Electrochromatographic column

Fig. 1 is a photograph of the device, and the assembled column consists of parts A, B and C, shown in Fig. 2. Part A is the actual Sephadex column, of length 1 m and I.D. 2.5 cm. To dissipate the heat produced by electrophoresis, the column has a cooling jacket for tap water. At the upper end of the column the glass is densely perforated along the whole of its circumference into a ringlet (*ca.* 1 cm high) of minute holes. Just under this ringlet a bowl is fused to the column. This bowl is filled with agar to a level above the ringlet. Over the agar (gel) is a buffer solution in which is a platinum wire (anode). The lower end of part A, with a sintered bottom, is joined with part B. The side tube of part B drains off the eluate. On the sintered glass (partition) in part B

is a layer of agar gel. Part B fits into a three-necked 500-ml flask (part C). One of the side necks is fitted with a rubber stopper through which a platinum wire (cathode) is inserted. The other side neck is an outlet for hydrogen evolved on the electrode. The flask is filled with dilute hydrochloric acid to trap the hydroxyl ions generated by electrolysis (sodium ions pass to the cathode from the column). The lower end of part B should reach deep below the level in the flask so that the bubbles evolved on the cathode cannot gather under the sintered partition.

Fraction 19S and the buffers employed

Fraction 19S was obtained from human serum by the standard technique of gel chromatography[1] on Sephadex G-200. It was concentrated to at least a half of its volume and dialysed against the buffer chosen for the electrochromatographic separation, *viz.*, 0.01 *M* Tris–HCl–0.1 *M* NaCl, pH 8.4, or 0.05 *M* $Na_2B_4O_7$ adjusted to pH 8.4 with hydrochloric acid.

Procedure

Part C is filled to about three-quarters of its volume with 0.1 *N* hydrochloric acid. Part B is inserted into part C and the hydrochloric acid is forced by a pressure difference to a level above the sintered partition. It is important that no air remain in the partition (ohmic resistance). The level of hydrochloric acid is lowered to about 2 mm above the partition and a hot 3% solution of agar in the chosen buffer (Tris or borate) is poured in to a level under the side-tube. Part B (joined with C) is now tilted with the side tube upwards so that more agar can be added without choking the side tube (to reduce the volume left above the gel) and the agar is allowed to form a gel. The same agar solution is poured into the bowl at the upper end of part A; its level should reach about 1 cm above the ringlet of holes. The space over the gel in part B and its side tube are filled with the buffer and the stop-cock is closed. The volume of buffer over the gel can be reduced further with the aid of glass beads. Part A is now joined with part B; no air should remain in the sintered bottom of part A or under it. The column is packed with Sephadex G-200, swollen in the buffer, to a level near to the end of the cooling jacket. The upper surface of the bed should be protected with a suitable porous material, *e.g.* a nylon net. About 8 ml of the concentrated fraction 19S is applied to the column. After the sample has entered the Sephadex bed, the buffer is added to such a level that it contacts the agar gel through the ringlet of holes. A portion of this buffer is poured onto the agar gel in the bowl and the column is closed with a rubber stopper through which are inserted two syringe needles. One needle is the inlet of buffer from a Mariotte flask and the other is attached to a piece of tubing on which is a clamp. With the aid of this tubing the buffer is introduced into the column from the Mariotte flask (a separatory funnel is useful for this purpose) by suction at the start of an experiment. The suction should be moderate so as not to tear off the agar gel in part B from the sintered glass. As soon as the buffer starts flowing into the column the open piece of tubing is compressed tightly with the clamp and the stop-cock on part B is opened. A suitable flow-rate, 8–12 ml/h, is adjusted by finding the appropriate elevation of the Mariotte flask. The upper (+) and the lower (—) platinum electrodes are connected to a potential difference of about 400 V. The buffer in the bowl of part A is continuously exchanged with a pump, and the lower end of part A and the upper end of part B are cooled with a wrap of wet cotton-wool.

The electrophoresis should accompany the gel chromatography for about 5 h (part B must be quite cool before IgM enters it). The first 60 ml of the eluate can be discarded, then 2-ml portions are collected and investigated spectrophotometrically for the presence of protein. The composition of the subfractions is examined by immunoelectrophoresis.

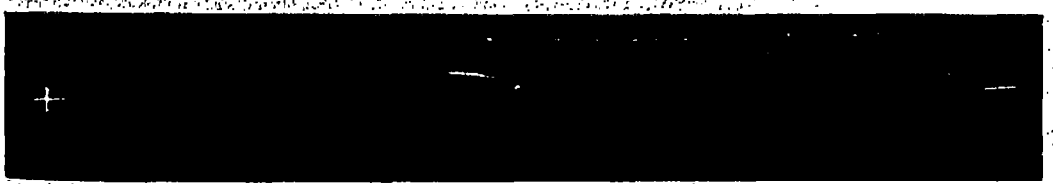


Fig. 3. Immunoelectrophoresis of the starting fraction 19S. From left to right: β_1-lipoprotein, α_2-macroglobulin and IgM. Horse serum against human serum proteins was used.

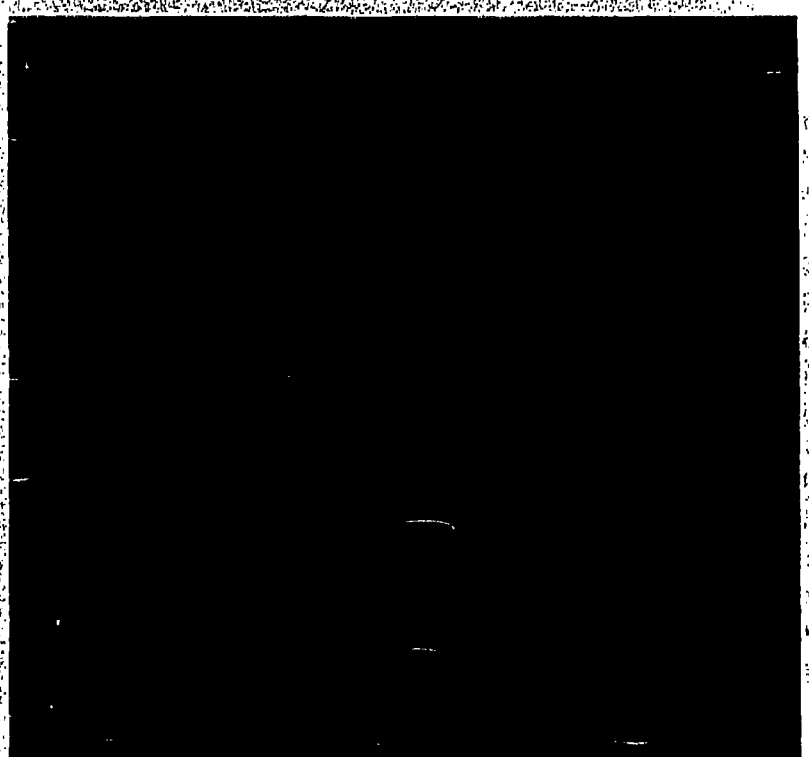


Fig. 4. Immunoelectrophoresis of the first subfractions from the electrochromatography of fraction 19S. Horse serum against human serum proteins was used.

RESULTS AND DISCUSSION

The immunoelectrophoretic pattern of the starting fraction 19S shows the presence of β_1-lipoprotein, α_2-macroglobulin and IgM (Fig. 3). Immunoelectrophoresis of the first subfractions obtained in the re-chromatography combined with electrophoresis demonstrates that virtually pure IgM is the first protein to leave the column (Fig. 4, top). This is closely followed by α_2-macroglobulin and β_1-lipoprotein, the advance of which through the column was retarded by electrophoresis. The identity of the individual proteins was verified with the respective monovalent sera.

In the normal gel chromatography of serum the maximum concentration of

IgM does not coincide with the top of the first peak (fraction 19S); it occurs at about three-quarters of the ascending part of the curve. This fact is a guide in considering which part of the 19S peak should be pooled as the starting material for the electrochromatographic isolation of IgM. The optimum pH for electrochromatography appeared to be 8.4, but a range of 8.2–8.8 seemed possible.

A drawback of the method is the low yield of pure IgM. However, the results suggest that with a stronger source of electric field the method might prove rewarding. Another expedient, although not very effective in the present case, is to extend the possible time of electrophoresis by reducing the flow-rate or discontinuing the gel chromatography for a period. The yield would further be raised by repeating the process with the eluate from the first electrochromatographic run, selecting the subfractions rich in IgM but contaminated with α_2-macroglobulin.

Compared with the two electrochromatographic columns recently proposed[11,12], the device described in this paper seems to be simpler in construction since no buffer reservoirs are attached to the column. A rather weak point, however, is part B, which allows little reserve for increasing the voltage applied. With a more efficient cooling of this part the separating power of the column would increase.

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