CHROM. 3340

SEPARATION OF MOUSE IMMUNOGLOBULINS ON THE BASIS OF WATER-SOLUBILITY ON SEPHADEX G-25

H. BAZIN, M.-C. SAVIN AND H. S. MICKLEM*

Laboratoire Pasteur de l'Institut du Radium, Paris V (France) and M.R.C. Radiobiological Research Unit, Harwell, Berks. (Great Britain)

(Received November 23rd, 1967)

SUMMARY

Whole mouse serum was placed on columns of Sephadex G-25 "Fine" or "Superfine" equilibrated with distilled water or 0.0001 *M* phosphate, and eluted with 0.01 *M* phosphate, pH 7.4, containing 0.15 *M* NaCl. Proteins were eluted in two peaks. The first emerged in approximately the void volume of the column; the second emerged with and slightly in advance of the salt front. IgM was almost entirely confined to the 2nd peak, which also contained 30–50 % of the IgG, 15–35 % of the IgA, some β-2C and occasionally small amounts of other non-immunoglobulin proteins. Most of the IgA, 50–70 % IgG, and sometimes small traces of IgM appeared in the 1st peak, which also contained the bulk of the non-immunoglobulin proteins. Further purification of the 2nd peak proteins was capable of yielding IgM free of contaminants detectable by immuno-diffusion. The best results were obtained with "Superfine"-grade Sephadex G-25.

INTRODUCTION

The differential solubility of serum proteins in distilled water or solution of low ionic strength has long been known, and permits the selective precipitation of euglobulins by dialysis. The advent of dextran gels (Sephadex) has made it possible to change the salt concentration of protein solutions with greatly increased speed and efficiency, and may facilitate the separation of proteins on the basis of solubility differences. This possibility has been to some extent explored by EPSTEIN AND TAN[1,2] and FJELLSTRÖM[3], who passed human serum through columns of Sephadex G-25 in solutions of low ionicity, and by PORATH[4] who employed a gradient of increasing concentrations of ammonium sulphate.

In the present paper we describe a simple method for fractionating mouse serum proteins by passage through a column of Sephadex G-25 equilibrated with distilled water or 0.0001 *M* phosphate, phosphate-buffered NaCl being used for elution. 'Euglobulins' are selectively retarded on the column. By means of this technique it is possible to obtain immunoglobulins free from the bulk of other serum proteins. In particular IgM may be obtained free of α2M-globulin in such a way that

* Present address: Department of Zoology, Edinburgh University, West Mains Road, Edinburgh, 9 (Great Britain)

it can be completely purified by subsequent passage through a column of Sephadex G-200; moreover, IgA can be obtained completely free of IgM.

METHODS

Sera

Mouse serum was obtained from adult A/H and C57BL/H animals. Usually the mice had previously received two subcutaneous injections of *Bordetella pertussis* vaccine (4×10^8 organisms), which were given to boost the serum immunoglobulin levels. The two injections were separated by a week and the mice were bled 10 days after the second.

Unless otherwise specified sera were separated and used within 5 h of bleeding.

Gel filtration

Sephadex G-25 (Pharmacia) was suspended in twice-distilled water and packed in columns of the following dimensions: 1.5×90 cm, 2.5×85 cm, and 5×100 cm. Initially "Fine" grade Sephadex (particle size 20–80 μ) was used, but for most of the work we employed "Superfine" (particle size 10–40 μ), the grade primarily intended by the makers for thin-layer chromatography. Before use, the bed was washed with at least 2 l of twice-distilled water or phosphate buffer (0.0001 *M*; pH 7.0).

Freshly prepared serum was applied to the top of the column—about 1.5 ml for a 1.5×90 cm column, 1–3 ml for a 2.5×85 cm column and 8–10 ml for a 5×100 cm column—and eluted with 0.01 *M* phosphate buffer, pH 7.4, containing 0.15 *M* NaCl. The flow rate was adjusted to between 10 ml/h and 30 ml/h according to the size of the column, although higher rates were obtainable even with superfine Sephadex and did not seem seriously to affect the separation.

Separation proceeded at room temperature. A column could be used several times provided that it was thoroughly washed with at least 3–4 l of 0.0001 *M* phosphate or distilled water.

Fractions of 2–6 ml were collected and their protein content was estimated spectrophotometrically at 280 mμ. Until the characteristics of a given column were established the specific resistance of each fraction was determined with a Phillips PR 9500 conductivity measuring bridge (cell constant = 1.3).

Fractions were pooled and concentrated by vacuum dialysis, dialysis against polyethylene glycol (mol. wt. 40,000: SERVA), or Aquacid I (Calbiochem).

Immunochemical analysis

Fractions were analysed by immunoelectrophoresis[5] in agar or agarose, by double diffusion in agar[6], and by single radial diffusion[7]. Antisera for the analysis were prepared in rabbits against whole mouse serum; mouse IgA; mouse IgM; mouse IgG1; mouse IgG2. The specific anti-mouse-Ig sera were prepared as described by BAZIN[8]. The same nomenclature for mouse immunoglobulins is employed as in previous papers[8,9], being based on that introduced by FAHEY *et al.*[10]

Protein concentration

The Biuret method as described by GORNALL and coworkers[11] was used for all protein determinations with a standard preparation of bovine serum albumin titrated by the Kjeldahl method.

RESULTS

The proteins of mouse serum emerged from a column of Superfine-grade Sephadex equilibrated with distilled water or 0.0001 *M* phosphate buffer, pH 7.0 in two well-separated peaks (Fig. 1). The first peak corresponded to the void volume of the column (*i.e.* that part of the total bed volume which lies outside the dextran gel granules), as measured by the passage of coloured Dextran 5000 (Pharmacia). The proteins of this peak had no tendency to precipitate even after prolonged storage. The second main protein peak emerged just before, and with, the salt front. The proteins in the earliest fractions of the second peak, *i.e.* those in which the salt concentration was lowest, tended to precipitate after a few hours in the tube at room temperature, but this could easily be prevented by the addition of a small amount of NaCl, or by using a refrigerated fraction collector.

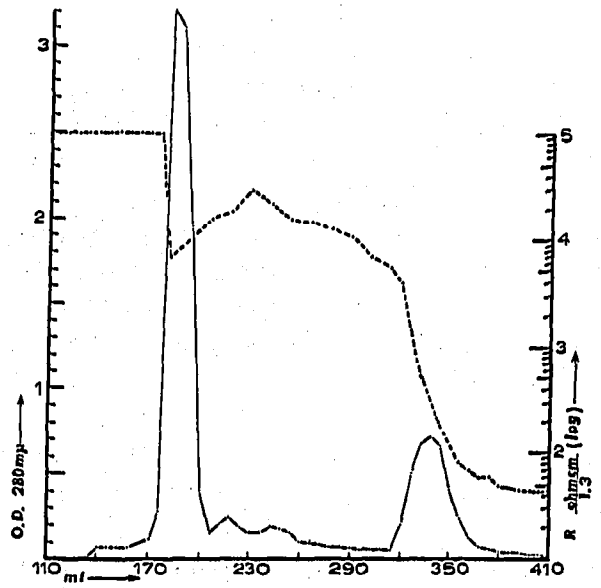

Fig. 1. Protein concentration (absorbance at 280 mμ) and specific resistance (cell-constant 1.3) of fractions eluted from a 2.5 × 85 cm column of Sephadex G-25 "Superfine" equilibrated with distilled water. 2 ml mouse serum eluted with 0.01 *M* phosphate, pH 7.4, 0.15 *M* NaCl.

At its best, the separation effected by fine-grade Sephadex was similar to the above. However, results with this grade were very much less reproducible. Some or even all of the water-insoluble proteins normally present in the second peak would often arrive with the first peak and form a dense cloudy precipitate in the collecting-tubes. The addition of NaCl to such tubes within one or two hours caused the precipitate to dissolve immediately.

Immunoelectrophoretic analyses showed that most of the serum proteins appeared in the first peak. These included pre-albumin, albumin, α_2-macroglobulin, transferrin, some IgG and many less easily recognizable proteins (Fig. 2a). It also contained some IgA. IgM was never detectable in immunoelectrophoretic analyses of the first peak, although traces were occasionally discernible in OUCHTERLONY plates and by the technique of single radial diffusion. The second peak consisted principally of immunoglobulins (IgM, IgG1, IgG2a, IgG2b) together with β-2C (part of the third component of complement), some IgA and sometimes traces of some other

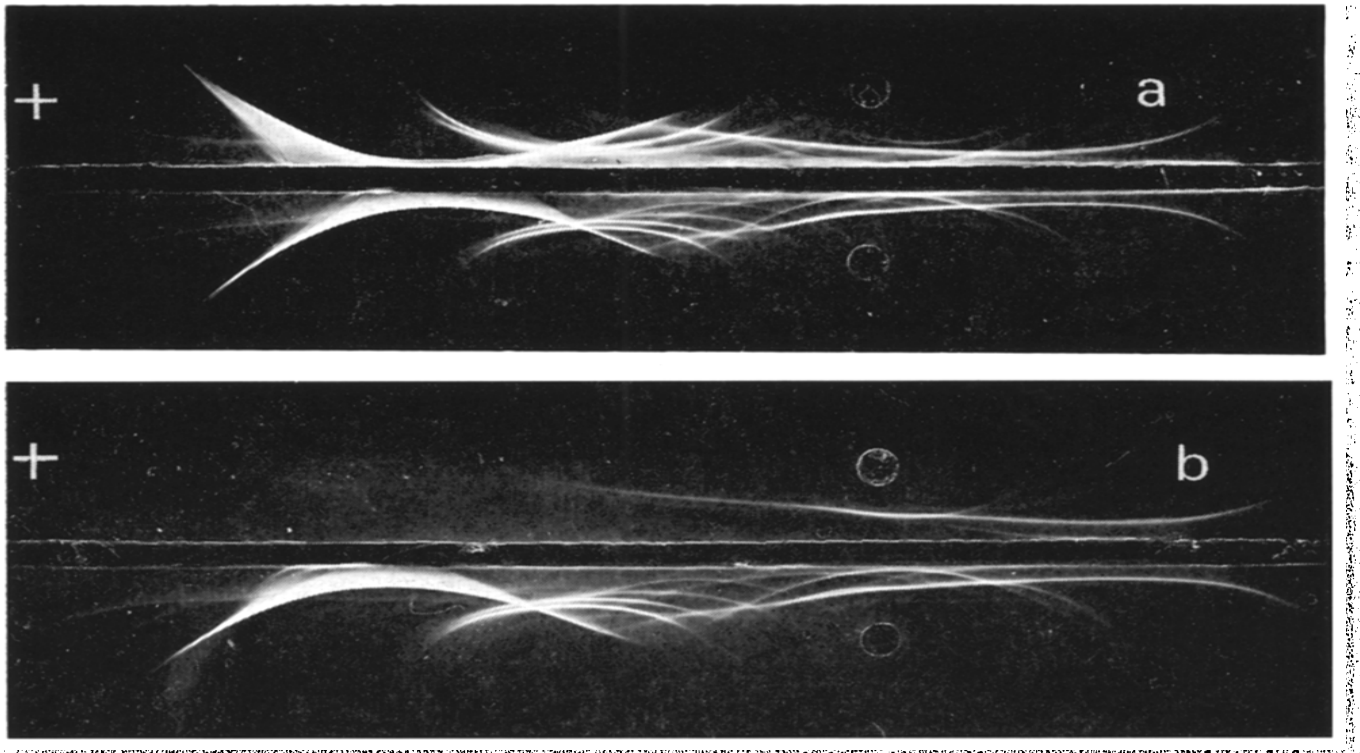

Fig. 2. Immunoelectrophoretic analysis of reconcentrated fractions of mouse serum eluted from a column of Sephadex G-25 "Superfine"; rabbit antiserum to whole mouse serum in troughs. (a) Upper well: 1st peak from column, lower well: unfractionated mouse serum. (b) Upper well: 2nd peak from column, lower well: unfractionated mouse serum.

unidentified proteins (Fig. 2b). The small amount of material eluted between the two main peaks (Fig. 1) consisted of proteins characteristic of the first peak.

Quantitative investigation, by single radial diffusion, of the immunoglobulins in the two peaks of five separated sera gave the approximate values shown in Table I.

TABLE I

DISTRIBUTION OF IMMUNOGLOBULINS BETWEEN THE TWO MAIN PROTEIN FRACTIONS ELUTED FROM SEPHADEX G-25 "SUPERFINE" COLUMNS, ESTIMATED BY SINGLE RADIAL DIFFUSION (5 POOLS OF SERUM)

	1st peak	*2nd peak*
IgA	65–85 %	15–35 %
IgG1	about 65 %	about 35 %
IgG2	50–80 %	30–50 %
IgM	traces or none	100 %

The best separations were obtained with fresh serum. Storage at —20° for three weeks or at 4° for one week resulted in trailing of the first peak.

The results were similar whether the column was equilibrated with distilled water or with 0.0001 *M* phosphate, pH 7.0. It is possible that manipulation of the buffering conditions and/or salt concentrations may produce further refinements in the technique. However, preliminary experiments indicate that columns equilibrated with 0.0001 *M* phosphate at pH 5.2 or 7.4 yield less useful separations.

Further purification of IgM on Sephadex G-200 followed by sucrose gradient ultracentrifugation

The second peak eluted from a G-25 column was concentrated and passed through a column of Sephadex G-200 equilibrated with 0.1 *M* Tris–HCl buffer, pH 8.2, containing 1 *M* NaCl. The same buffer was used for elution. The first half of the first peak so obtained consisted of IgM as judged by immunoelectrophoresis or double diffusion against a rabbit serum against whole mouse serum (Fig. 3). The yield of the recovered IgM was 15–20 % of the quantity in the original serum before all chromatography. It was sometimes contaminated with a small amount of α_2-lipoprotein. However, virtually complete purification, as judged from OUCHTERLONY plates, was obtained by subsequent ultracentrifugation on a sucrose gradient as described by ADLER[12], without further loss of IgM.

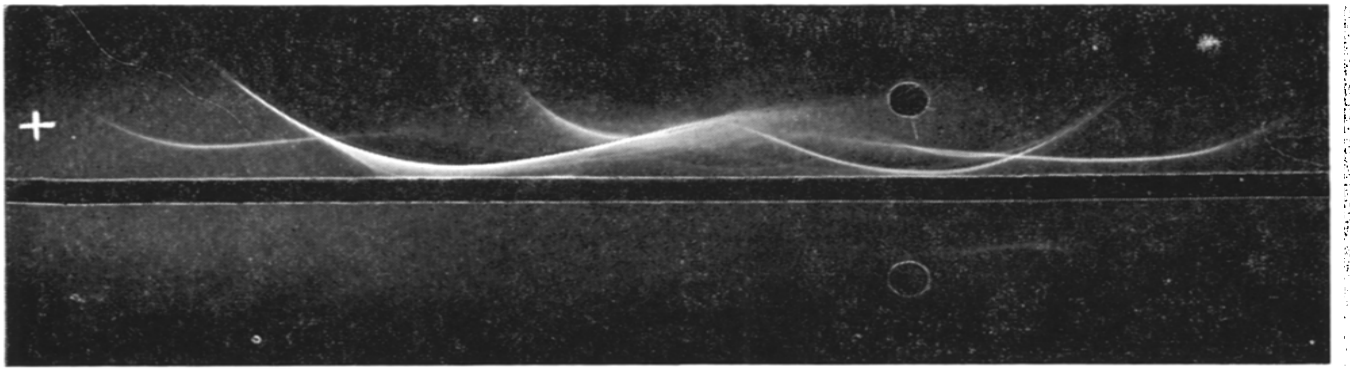

Fig. 3. Purification of mouse IgM. Rabbit antiserum to whole mouse serum in trough. Upper well: unfractionated mouse serum, lower well: IgM prepared from 2nd peak of a G-25 "Superfine" column by filtration through Sephadex G-200 (concentration of protein: 12 mg/ml).

DISCUSSION

In principle the technique described resembles ordinary dialysis against distilled water. Proteins move down the Sephadex G-25 column faster than do salts until they reach a zone of such low ionicity that some begin to precipitate. Once precipitated, the water-insoluble proteins may be arrested until redissolved at the arrival of the saltfront. This process may be envisaged as occurring repeatedly down the length of the column, with the euglobulins being continually precipitated and redissolved within a narrow zone of low ionicity. The critical salt concentration at which the precipitates begin to redissolve corresponds to a measured resistance of about 5,000 ohms/cm (representing an approximately 0.002 *M* solution of NaCl). However, not all the proteins remain stably in solution at this ionicity, since, as noted above, reprecipitation tends to occur unless the ionic strength is raised.

The critical ionicity zone may well not be the same for all the proteins in the second peak. During elution of the second peak the ionicity of the eluate was rising rapidly. Possible differences between proteins emerging at the beginning and end of the second peak were not investigated.

The two main advantages of the present method over standard dialysis are, first, that proteins are not held for protracted periods in the precipitated state, and second, that they have no opportunity to form large aggregates. Both factors should tend to reduce denaturation of the molecules. It is possible that reversible adsorption

of some protein molecules onto the surface of the Sephadex granules[13–15] may have been a minor complicating factor; such a phenomenon could account for the gradual emergence of a small quantity of protein between the first and second main peaks, and for the occasional contamination of the second peak with small amounts of material characteristic of the first peak.

The best and most reproducible results were obtained with Superfine-grade Sephadex. This may be due to a more complete arrest of fine protein precipates by the smaller size dextran particles.

The method described effects, simply and rapidly, separations which can usefully be employed in the purification of immunoglobulins. In particular, since α_2-macroglobulin appears entirely in the first peak, the method can be used in conjunction with Sephadex G-200 to prepare nearly pure IgM.

Most of this work was performed while one of us (H.B.) was a visiting worker at the Medical Research Council's Radiobiological Research Unit, Harwell, and was in receipt of a grant from Euratom.

REFERENCES

1 W. V. EPSTEIN AND M. TAN, *J. Chromatog.*, 6 (1961) 258.
2 W. V. EPSTEIN AND M. TAN, *J. Lab. Clin. Med.*, 60 (1962) 125.
3 K.-E. FJELLSTRÖM, *Acta Pathol. Microbiol. Scand.*, 54 (1962) 439.
4 J. PORATH, *Nature*, 196 (1962) 47.
5 P. GRABAR AND P. BURTIN, *Immunoelectrophoretic Analysis*, Elsevier, Amsterdam, 1964.
6 O. OUCHTERLONY, *Progr. Allergy*, 6 (1962) 30.
7 G. MANCINI, J. P. VAERMAN, A. O. CARBONARA AND J. F. HEREMANS, *Protides of the Biological Fluids (11th Colloquium, Bruges, 1963)*, 11 (1964) 370.
8 H. BAZIN, *Ann. Inst. Pasteur*, 111 (1966) 544.
9 H. BAZIN, *Ann. Inst. Pasteur*, 112 (1967) 162.
10 J. L. FAHEY, J. WUNDERLICH AND R. MISHELL, *J. Exptl. Med.*, 120 (1964) 223, 243.
11 A. G. GORNALL, C. J. BARDAWILL AND M. M. DAVID, *J. Biol. Chem.*, 177 (1949) 751.
12 F. L. ADLER, *J. Immunol.*, 95 (1965) 26.
13 B. GELOTTE, *J. Chromatog.*, 3 (1960) 330.
14 A. N. GLAZER AND D. WELLNER, *Nature*, 194 (1962) 862.
15 F. MIRANDA, H. ROCHAT AND S. LISSITSKY, *J. Chromatog.*, 7 (1962) 142.