

THE PURIFICATION OF HUMAN POLYCLONAL IgE BY IMMUNOSORPTION

R. C. AALBERSE, H. G. J. BRUMMELHUIS and E. E. REERINK-BRONGERS

Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, P.O.B. 9190, Amsterdam, The Netherlands

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Abstract—Polyclonal IgE is purified by incubation with insolubilized anti-IgE antibodies and subsequent elution at low pH. The best results are obtained when isolated anti-IgE antibodies are coupled to cellulose, and when gel filtration is used for neutralization. Using a mixture of anti-IgE cellulose and Sephadex G-25, the dissociated IgE is separated by gel filtration from the pH 2.8 elution buffer immediately after dissociation from the cellulose-bound antibodies.

Three steps precede this final procedure:

(a) absorption of a crude anti-IgE serum with insolubilized human serum proteins;
(b) partial purification of IgE by elution from an immunoglobulin fraction of the anti-IgE serum coupled to cellulose;

(c) isolation of anti-IgE antibodies by elution from partly purified IgE coupled to cellulose.

The eluted IgE gives a reaction of complete identity with myeloma protein ND; its sedimentation constant is approximately 7.6 S.

INTRODUCTION

In the radio-allergosorbent test (RAST) IgE adsorption to cellulose-bound allergen is demonstrated by means of radioactive anti-IgE antibodies (Wide *et al.*, 1967).

In our first trials we found that an isotope-labelled immunoglobulin fraction of the available anti-IgE antiserum gave unsatisfactory results, and that isolation of the anti-IgE antibodies by adsorption to and elution from IgE was necessary. IgE myeloma protein would have been most suitable for this purpose, but only three such proteins have been described (Fishkin *et al.*, 1972; Johansson and Bennich, 1967; Ogawa *et al.*, 1969). We therefore had to isolate IgE from plasma with a high level of polyclonal IgE. IgE-isolation by a combination of salt fractionation, ion exchange chromatography and gel filtration appeared inefficient. Ito *et al.* (1969) were able to adsorb reagenic activity using an ethylchloroformate polymerized anti-IgE serum. A small part (6 per cent) of the adsorbed activity was recovered upon elution with a pH 2.2 buffer. No data on the purity of the desorbed material were given.

Bennich and Johansson (1971) purified anti-IgE antibodies by adsorption to and desorption from IgE myeloma protein coupled to Sepharose. The purified antibodies were then coupled to Sepharose and used to purify polyclonal IgE. The adsorbed IgE was desorbed either by acid elution or by 3.5 M thiocyanate elution.

In this paper a similar purification of IgE by immunosorption will be described, but in this procedure no myeloma protein is needed.

MATERIALS AND METHODS

IgE

Citrated plasma with a high IgE content was collected from two patients with tropical parasitic infections by means of plasmapheresis. One patient, Ki, suffering from a *Schistosoma mansoni* infection, had an IgE level of 180,000 U/ml; the other, Ch, with a tropical eosinophilia probably due to a microfilaria infection, had an IgE level of 50,000 U/ml. The plasma was defibrinated by addition of thrombin (Topostasine, Roche).

Anti-IgE serum

An anti-IgE serum was prepared by immunizing a sheep with the IgE containing fractions of plasma Ki, isolated according to Ishizaka and Ishizaka (1966). In this study three different batches of the sheep antiserum were used; the unabsorbed batches were coded up1, up4 and up7. After absorption of the non-anti-IgE antibodies by adding normal human serum, which contained hardly any IgE, the batches were coded p1, p4, and p7 respectively. # up1 and up7 appeared to be monospecific for IgE after absorption with vol 1 of normal human serum, as tested by agar diffusion; # up4 had to be absorbed with vol 3 of normal human serum.

A sheep antiserum prepared against the Fc fragment of IgE myeloma protein ND was a gift from Dr. S. G. O. Johansson, Uppsala.

Single radial diffusion (SRD)

2% agar in phosphate buffered saline (PBS, 0.14 M NaCl, 0.01 M phosphate pH 7.4) was

mixed with antibody (Mancini *et al.*, 1965) or antigen (Stiehm, 1967). Agar layers with a thickness of 1.6 mm were prepared. Antibody containing agar was used for the IgE, IgG and IgM assays. The WHO reference preparation 68/341, IgE content 10,000 U/ml, was used as the IgE standard; as a reference for IgG served the standard serum of Behringwerke No. 469, IgG content 9.9 mg/ml.

Antigen containing agar was used for the estimation of antiserum potency. Although not only the biological activity of IgE, but also the antigenic structure is heat labile, satisfactory results were usually obtained if the plasma-agar mixture was cooled as rapidly as possible. The anti-IgE potency was expressed in arbitrary potency units (PU). Anti-IgE serum # up4 was used as a reference; it was assumed to contain 1 PU/ml. Diffusion of a 3.5 μ l sample of this antiserum into agar containing 0.8 ml IgE plasma Ch per 12 ml resulted in a precipitation ring with a diameter of 6 mm.

The anti-IgE/IgE precipitation ring could easily be identified by comparison with a control agar plate which contained normal serum instead of serum Ch. The validity of this procedure was proved by ring fusion of absorbed and unabsorbed anti-IgE serum. Anti-light chain antibodies did not influence the diameter of the IgE precipitation ring. Anti-IgE serum # p4, anti-IgE potency 0.25 PU/ml, had to be diluted 1:3 to precipitate at equivalence with the WHO reference preparation 68/341.

Immuno-electrophoresis was performed for 50 min at 8 V/cm. 1.3% Agar (Difco Noble) in 0.04 M barbitone buffer, pH 8.6, was used. 10 μ l sample was tested.

Analytical ultracentrifugation was carried out with 12 mm standard cells at 20°C in a model E Spinco ultracentrifuge at 54,780 rev/min.

Insolubilization of proteins

1. *Polymerization* was performed according to Avrameas and Ternynck (1969) with glutardialdehyde (GDA). The gel obtained was homogenized and washed.

2. *Coupling to cellulose*. Cellulose was substituted with m-nitrobenzyl-oxymethylene groups, reduced with dithionite and treated with Cu(OH)₂ dissolved in ammonia (Gurvich *et al.*, 1961; Brummelhuis, 1972). The nitrogen content of the batch was 0.165 gN/100 g aminocellulose. After diazotization the cellulose was brought to pH 8.6 (0.05 M borate buffer) and protein was added. The amount of protein bound after incubation for 20 hr or more at 4°C was calculated from the protein content of the supernatant. Residual diazo groups were blocked with beta-naphthol.

IMMUNOSORPTION

A column was partly filled with Sephadex G-25 (coarse). A mixture of cellulose-bound antigen or

antibody and Sephadex G-25 (2–8 g dry-weight Sephadex/100 mg cellulose) was poured on this column.

The Sephadex was added not only as a 'solid diluent', but also as a molecular sieve (see below).

The procedure, carried out at room temperature, was as follows: 10–50 ml antiserum (or serum), two bed volumes PBS, one bed volume elution buffer, –133 mM glycine, 104 mM citric acid, 154 mM NaCl, (pH 2.8) were successively percolated through the column.

The antigen-antibody complex dissociated in the elution buffer; the desorbed protein was separated from the elution buffer immediately afterwards because of the molecular sieving effect of Sephadex G-25.

The fraction that left the column at a neutral pH, immediately followed by a sharp drop of pH, thus contained the eluted protein (Fig. 1).

To demonstrate specific adsorption of IgE on insolubilized anti-IgE the IgE concentration in the plasma was measured before and after incubation. To correct for dilution and non-specific adsorption the concentration of a non-related protein was also measured; for this purpose IgM was chosen arbitrarily. The purest IgE preparations were obtained in four steps:

(A) Absorption of crude anti-IgE serum with insolubilized normal human serum. The absorbed antiserum was coded A1.

(B) Partial purification of IgE by elution from A1 coupled to cellulose. This resulted in the IgE preparations coded B1 and B2.

(C) Isolation of anti-IgE antibodies by elution from the partly purified IgE preparation B1 coupled to cellulose. This resulted in an anti-IgE preparation coded C1.

(D) Elution of IgE from preparation C1 coupled to cellulose. These final IgE preparations were coded D1 and D2.

(adA) Absorption of crude anti-IgE serum with insolubilized normal human serum

A 2 M ammonium sulphate precipitate from 300 ml normal human plasma was insolubilized by addition of GDA. The polymerized material was used to absorb 50 ml anti-IgE serum # up4. The supernatant and the neutral washings were pooled and concentrated by half saturation with ammonium sulphate. This anti-IgE preparation still contained contaminating antibodies, mainly reacting with transferrin. No antibodies to IgG were demonstrable.

(adB) Elution of IgE from preparation A1 coupled to cellulose

30 ml A1 were added to 800 mg diazotized cellulose, which bound 24 per cent of the protein present.

Approximately 700 mg of this anti-IgE cellulose

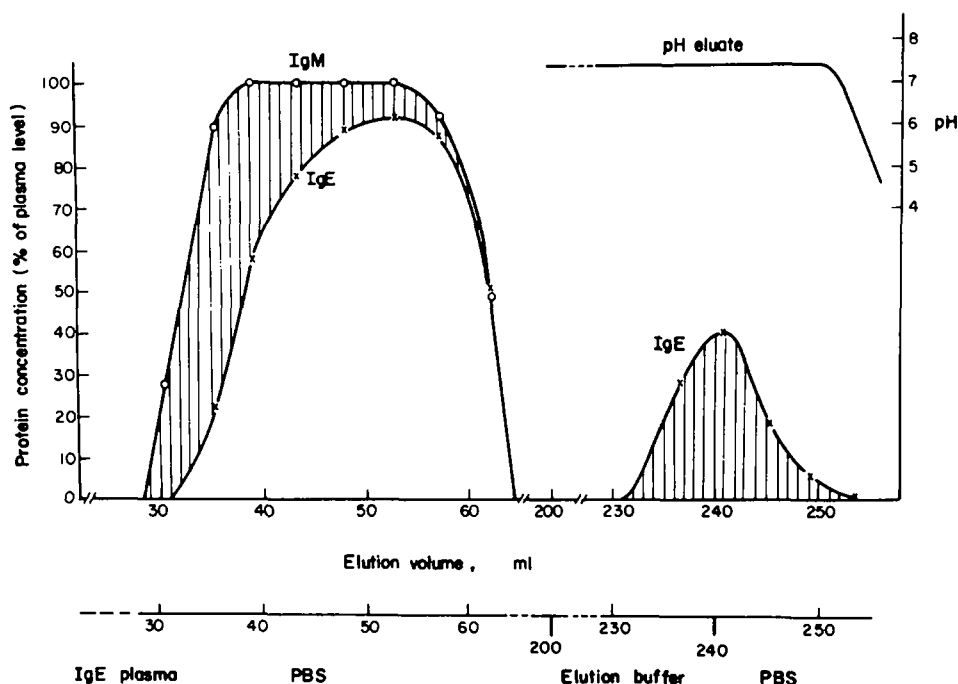


Fig. 1. Fractionation of IgE plasma Ch with the aid of an anti-IgE preparation (purified antibodies) coupled to cellulose and mixed with 5 g Sephadex G-25. This mixture was added to a column of 10 g Sephadex G-25, final column size 55×1.2 cm. The amount of anti-IgE insolubilized was 0.7 PU. In the eluate the relative concentrations of IgM and IgE were measured. The elution pattern of IgM was taken as a prototype of elution of a non-adsorbed protein. The difference in IgM and IgE patterns at neutral elution is due to specific IgE adsorption. The hatched area on the left, therefore, represents the amount of adsorbed IgE (360,000 U IgE). The elution pattern of IgE, desorbed by lowering the pH, is reproduced at the right. Note that IgE has been eluted just before the pH drops. The hatched area on the right represents the yield of purified IgE (200,000 U IgE). —o—o— IgM concentration in column effluent, —x—x— IgE concentration in column effluent, ——— pH of column effluent.

was mixed with 35 g Sephadex G-25; this mixture was added to a column of 15 g Sephadex G-25 to make a column of 20×4 cm. The IgE-rich plasma Ch was fractionated on this column. The first 75 ml obtained after starting acid elution were discarded. The following fractions were pooled until the pH had fallen to 3.5. Fractions with a lower pH were discarded. The eluates of the first 10 runs were pooled, concentrated by positive pressure dialysis (Diaflo UM-10) and dialyzed against 0.05 M borate buffer pH 8.6. The yield of 1.4 ml contained 1,100,000 U IgE/ml. (IgE preparation B1).

The next six eluates were pooled, concentrated and dialyzed against PBS. The yield of 9.7 ml contained 69,000 U IgE/ml (IgE preparation B2).

(adC) Elution of anti-IgE antibodies from preparation B1 coupled to cellulose

1.1 ml B1 was added to 100 mg diazotized cellulose, which bound 65 per cent of the protein present. The IgE cellulose was used in a column of 15×1.5 cm.

Anti-IgE serum # p4 (# up4 made monospecific

by addition of vol 3 of soluble normal human serum) was fractionated on this column. A pool of 20 eluates was concentrated to approximately 10 ml.

(adD) Elution of IgE from preparation C1 coupled to cellulose

Four millilitres of C1 were added to 70 mg diazotized cellulose. The anti-IgE containing cellulose was used in a column of 26.5×2.0 cm. IgE plasma Ch was fractionated on this column. A pool of the first 10 eluates was concentrated to 10 ml containing 240,000 U IgE/ml, and called D1; a pool of the next eight eluates was concentrated and dialyzed against PBS; the resulting 4.0 ml were filtered through 0.45μ pore Swinnex-25 (Millipore Co.); the IgE-content of this preparation D2 after filtration was 800,000 U/ml.

RESULTS

The results are summarized in the flow diagram (Fig. 2) and in Tables 1 and 2. The relative purity of the IgE preparations can be estimated from the

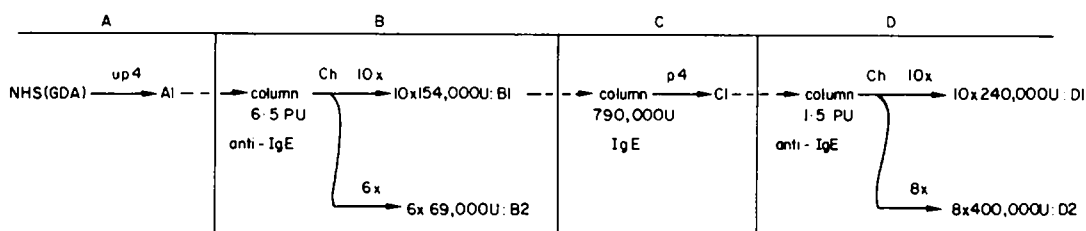


Fig. 2. Flow diagram summarizing the results of the procedure used.

Table 1. Anti-IgE preparations used for IgE-purification

Anti-IgE preparation	OD280	Anti-IgE potency ^a	PU/ml OD280
A1	33	1.10	0.033
C1	2.0	0.78	0.39

^aAnti-IgE potency is expressed in potency units (PU) assuming that anti-IgE serum # up4 contains 1 PU/ml.

IgE/IgG ratios or from the IgE/O.D. 280 ratios, as well as from the immunoelectrophoretic analysis against a polyvalent antiserum (Fig. 3a). In Fig. 4 preparation D2 is tested seven times more concentrated. Both monoclonal and native polyclonal IgE have an alpha-2 electrophoretic mobility. Most of the isolated IgE had a similar or somewhat higher mobility (Fig. 3b), but sometimes one or more components with a lower mobility were present as well (preparation B1 and B2, Fig. 3b; preparation D2 when tested in a high concentration, Fig. 4). In double diffusion experiments IgE eluted from isolated antibodies (D experiments) gave a reaction of complete identity with native IgE or myeloma IgE (a gift from Dr. H. Bennich, Uppsala) as illustrated in Fig. 5; but IgE isolated in the B-coded

experiments contained material antigenically deficient compared to native IgE (Fig. 6).

The ultracentrifugation run of preparation D2 (O.D. 280 = 5.3) showed a major component sedimenting at 7.3 S, and a minor component sedimenting at 13 S (Fig. 7). These values could not be extrapolated to zero protein concentration, as only one single run could be made due to the limited quantity of the material available. If we assume a concentration dependency comparable to IgG, the corrected sedimentation coefficient is approximately 7.6 S for the main component.

DISCUSSION

In preliminary experiments two other procedures were tested: a batch procedure and GDA-insolubilization of the antibodies.

Batch procedure

When a gammaglobulin fraction of the anti-IgE serum was used instead of purified antibodies, the yield of IgE was higher in a batch procedure than in a column procedure: 42,000 vs 14,500–22,000 U IgE/PU anti-IgE insolubilized. However, there was more contamination present, i.e. an IgE/O.D. 280 value of 45,000 U was found after a batch

Table 2. IgE-preparation

Experiment	Anti-IgE insolubilized PU ^a	IgE yield ^b U/PU	IgE/OD280 U	IgE/IgG ^c U/ng
B1	6.5	22,000	61,000	0.72
B2	6.5	14,500	75,000	0.53
D1	1.5	160,000	120,000	2.35
D2	1.5	250,000	151,000	2.0

^aInsolubilized anti-IgE potency was calculated as the potency of the corresponding amount of soluble anti-IgE as estimated by single radial diffusion; it was expressed as PU (potency units) assuming that anti-IgE serum # up4 contained 1 PU/ml.

^bThe IgE yield was calculated by multiplying the final IgE content of the eluate (after concentration and dialysis) by the final volume and dividing the results by the number of runs.

^cThe IgG content was estimated by single radial diffusion and expressed in ng/ml, assuming that the standard serum of Behringwerke No. 469 contained 9,900,000 ng/ml.

The IgE/IgG ratio in the starting material (serum Ch) was 0.0038 U/ng.

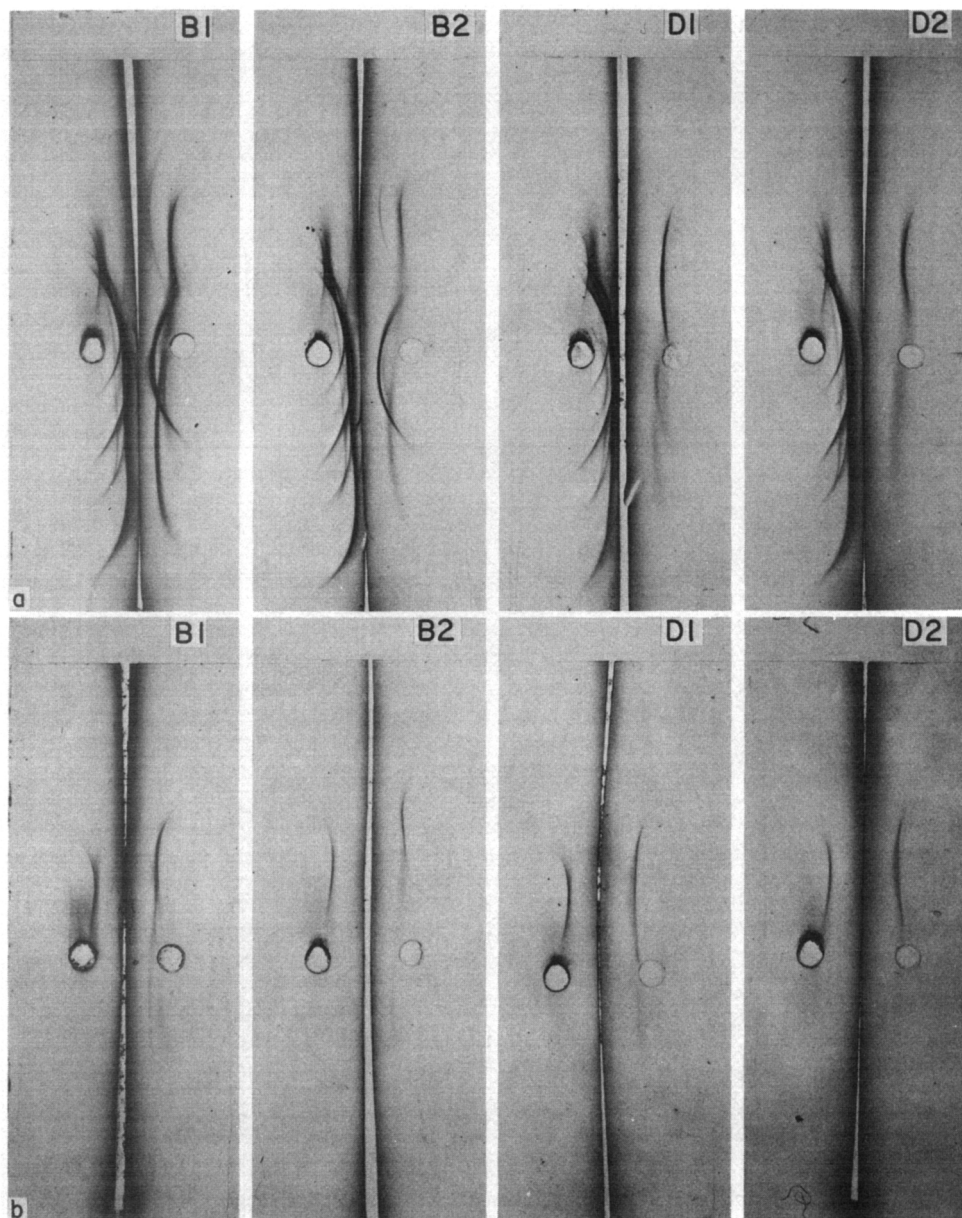


Fig. 3. Immunoelectrophoresis of the IgE preparations compared to the starting material Ch. B1 (IgE: 230,000 U/ml; O.D. 280 = 3.8), B2 (IgE: 69,000 U/ml; O.D. 280 = 0.92), D1 (IgE: 240,000 U/ml; O.D. 280 = 2.0), D2 (IgE: 115,000 U/ml; O.D. 280 = 0.76). 10 μ l samples are tested. The trough contains unabsorbed anti-IgE # up7 (Fig. 3a) or the same antiserum after absorption with vol 1 normal human serum (# p7, Fig. 3b). The anode is at the top.

procedure vs 61,000–75,000 U after a column procedure.

When purified antibodies were used the results of the batch procedure and the column procedure were comparable as far as the IgE yield was concerned, but again there was more contamination present after a batch procedure: an IgE/O.D. 280

value of 61,000 was found in the batch procedure versus 120,000–151,000 U in the column procedure.

GDA-insolubilization

Polymerization or copolymerization was very easy to perform. It appeared, however, that GDA-polymerized antibodies had a lower antigen-binding

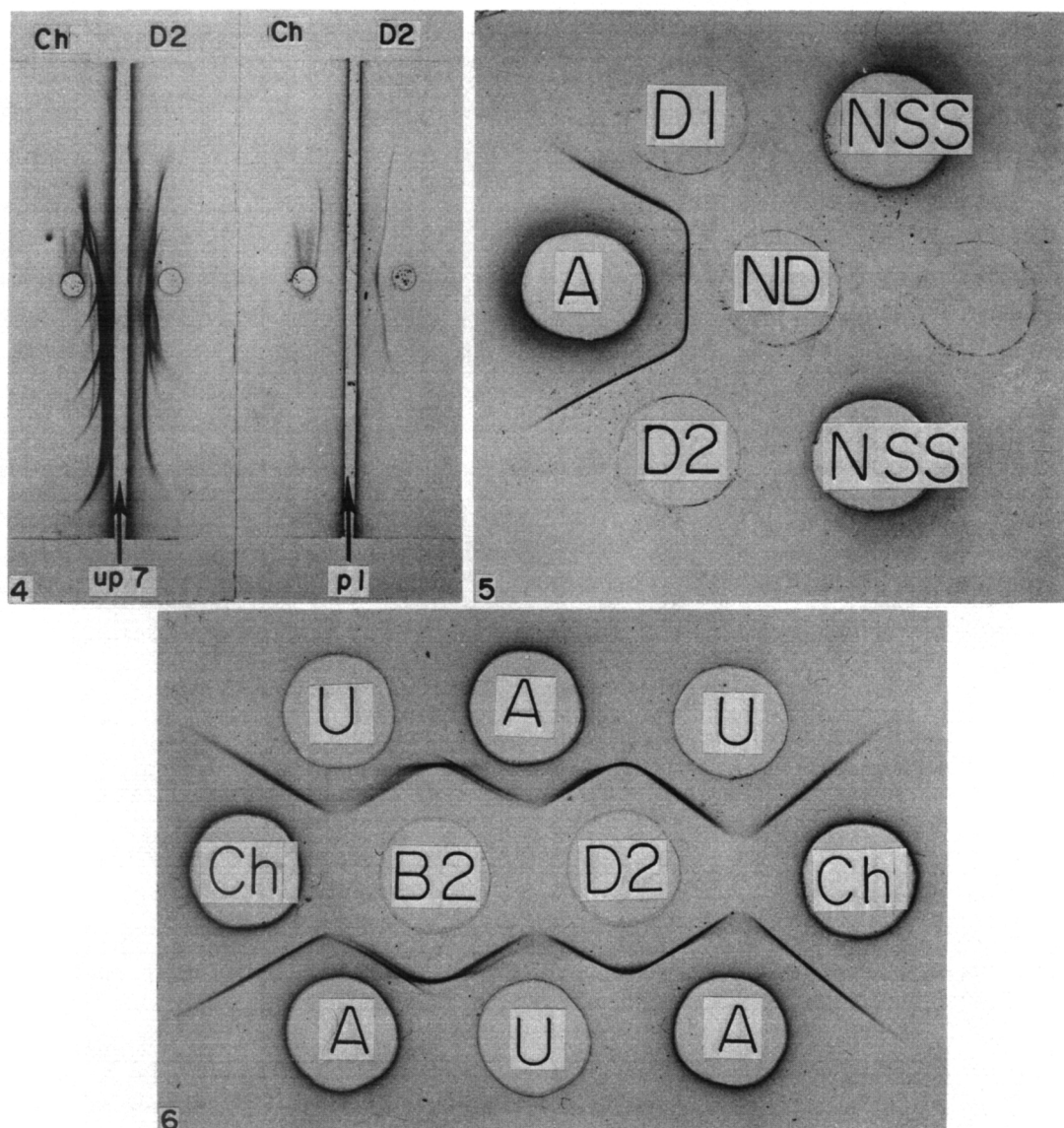


Fig. 4. Immunoelectrophoresis of IgE-preparation D2 tested seven times more concentrated than in Fig. 3 (IgE: 800,000 U/ml; O.D. 280: 5.3). The IgE precipitation line in the $\alpha 2$ region is formed in a large antigen excess. The trough contains unabsorbed anti-IgE serum # up7 or absorbed anti-IgE serum # p1.

Fig. 5. Double diffusion analysis of two polyclonal IgE preparations, D1 and D2, compared to an IgE myeloma protein preparation (IgND). The optical density at 280 nm of all three preparations was 0.28. The antiserum (A) was the sheep antiserum raised against polyvalent IgE (anti-IgE # p1). To exclude the possibility that preparations D1 and D2 contained heterophile antibodies to sheep immunoglobulin instead of IgE, two holes were filled with normal sheep serum (NSS). The reaction was negative.

Fig. 6. Double diffusion analysis of two polyclonal IgE-preparations B2 and D2 compared to the starting material (Ch.). The anti-IgE serum provided by Dr S. G. O. Johansson is coded U; anti-IgE serum # p7 is coded A.

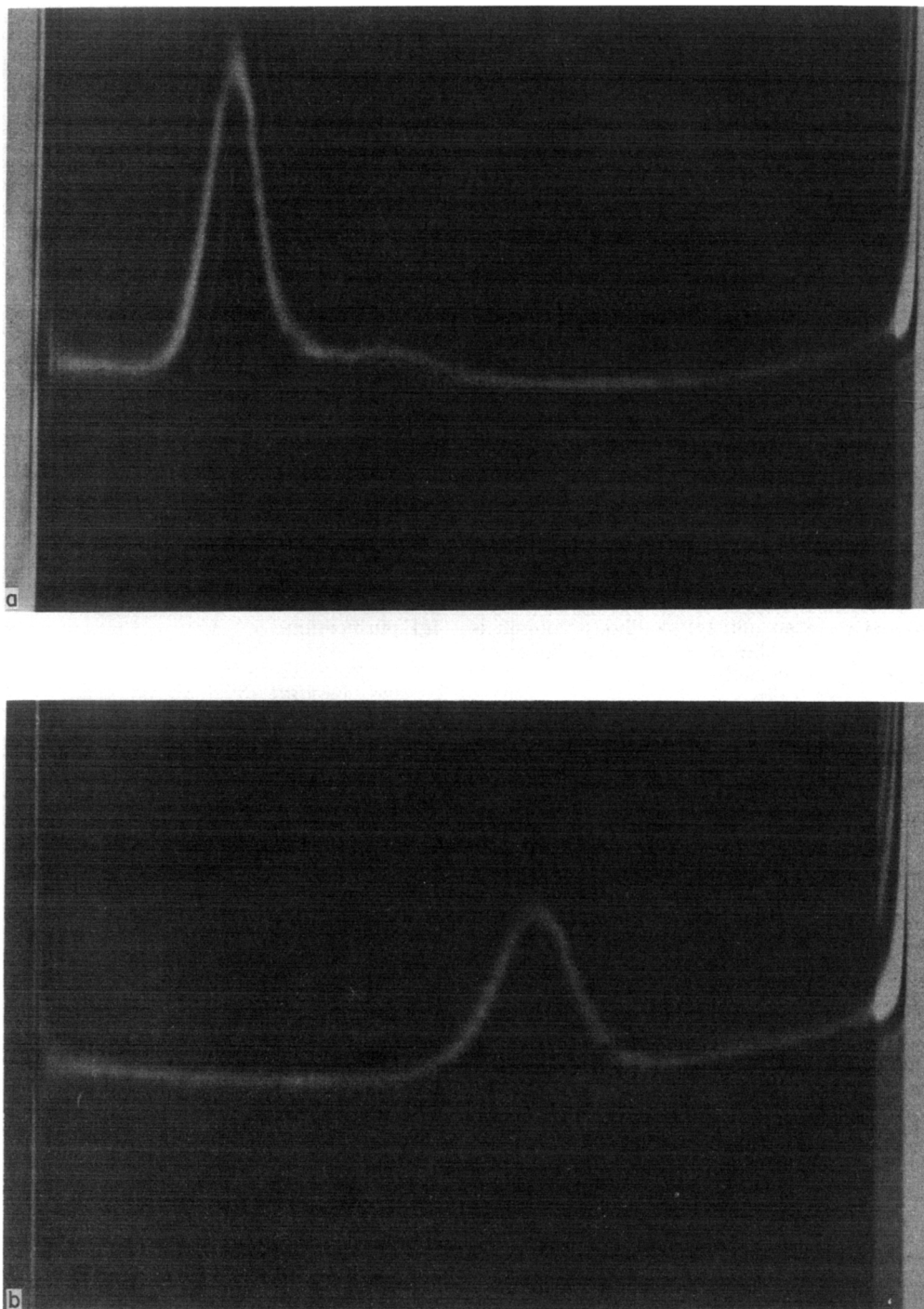


Fig. 7. Ultracentrifugation pattern of IgE preparation D2, O.D. 280 = 5.3 IgE content 800,000 U/ml in PBS. The calculated sedimentation constant was 7.3 S; a minor component sedimented at 13 S (non-corrected values). At a similar protein concentration the S value for IgE (ND) was about 7.8 (Bennich, 1971). (a) 20 min (b) 60 min.

capacity compared to cellulose-coupled antibodies; moreover, the purity of the material desorbed was low: IgE/O.D. 280 values of 12,000–22,000 U were found.

Therefore, a column procedure in which cellulose-bound antibodies were used, was considered to be the most suitable.

Comparison of B- and D- experiments: qualitative considerations

Changes in both antigenic composition and electrophoretic mobility were found in the B-preparations.

B1 and B2 contained components antigenically deficient compared to native IgE (Fig. 6). This may be due to partial denaturation, aggregation or fragmentation. No spurring occurred when the D-preparations were tested; complete identity reactions were found with both monoclonal and polyclonal IgE.

Changes in electrophoretic mobility were, however, apparent even in the D-preparations, especially when tested in high concentrations (Fig. 4).

The biological activity of the purified material has not yet been tested. The antibody activity of IgE eluted from an anti-IgE antibody column is largely retained as shown in the radio-allergosorbent test (to be published).

Quantitative considerations

Approximately 20 per cent of the O.D. 280 of the B-preparation can be ascribed to the contaminating antibodies to transferrin in anti-IgE preparation A1. The IgG contamination of especially the D preparations (Table 2) is much higher than the contamination by e.g. albumin. This indicates selective absorption of IgG. By means of agar diffusion no precipitating antibodies to IgG could be demonstrated in either anti-IgE preparation A1 or C1. The IgG contamination could result, however, from the presence of non-precipitating antibodies; furthermore, this contamination may represent human anti-sheep antibodies, although no such antibodies could be demonstrated by agar precipitation.

When purer IgE is desirable contaminating antibodies should be excluded more rigorously; to the IgE plasma some sheep immunoglobulin can easily be added to absorb possible heterophile antibodies; moreover, the resulting IgE preparation can be purified by the addition of anti-IgG immunosorbents. The purity of the IgE preparations we obtained is difficult to assess. It is generally agreed that one IgE unit is more than 1 ng, but the exact value is not yet known. A purified IgE (ND)-myeloma preparation, kindly provided by Dr. H. Bennich, Uppsala, had an IgE/O.D. 280 value of 132,000 U in the assay used in this investigation, which remains within the range of values obtained

for our D preparations. Bazaral and Hamburger (1972) used another purified IgE myeloma protein (PS) as a standard. In their radioimmunoassay they found 2.4 U IgE/ng IgE (PS). This corresponds to an IgE/O.D. 280 value of 293,000 U.

As presumably none of our IgE preparations are completely free of aggregated or fragmented proteins SRD is not a reliable technique for IgE or IgG quantification. This is most apparent when the B-preparations are tested for IgE: a faint precipitation ring outside the major ring is then found. The IgE concentrations reported are based on the diameter of the strongest precipitation ring.

Anti-IgE potency values obtained from reversed type SRD experiments should also be looked at critically: the ring size is not only related to the antibody content, but also inversely related to the number of antigenic determinants recognized by the antiserum, i.e. by the Ab/Ag ratio at extreme antibody excess (Trautman *et al.*, 1971). Furthermore, the ring size may be dependent on the mol. wt (apart from IgG, IgM, aggregates and antibody fragments may occur).

Nevertheless, the results demonstrate that affinity chromatography using acid elution in a gelfiltration medium is a very useful method for IgE purification.

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