

## Effect of Electrolytes and of Distilled Water on Antigen-Antibody Complexes

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Specific immune precipitates dissolve in concentrated solutions of alkali-metal halides, and of alkaline-earth-metal halides and thiocyanates. The quantity of protein dissolved depends on the nature of the antigen-antibody system, on the proportion of the antigen in the precipitate, and on the avidity of the antibody. The extent of solubilization is a function of the temperature, of the volume of solution used and of the concentration of the ions in the solution, and also depends on the nature of these ions. The dissolving power of bivalent cations is greater than that of monovalent ones, and is as follows:  $Mg^{2+} > Ba^{2+} > Ca^{2+} > Sr^{2+}$ . Antigen-antibody complexes and free antibodies, but no free antigen, are detected in supernatants of specific precipitates dissolved in solutions of electrolytes of low ionic strength. Antigen-antibody complexes, free antibodies and also free antigen are detected in supernatants of specific precipitates dissolved in solutions of electrolytes of high ionic strength. Comparable results are obtained when the electrolyte solutions are studied for their effect on the bonds formed between an antibody and its corresponding immunosorbent. Moreover, in the latter case, 50% of the fixed antibodies could be recovered by elution with distilled water.

Heidelberger and his collaborators have reported that the formation of specific precipitates by pneumococcal polysaccharide and homologous antibody are inhibited by concentrated solutions of sodium chloride (Heidelberger, Kendall & Teorell, 1936). This observation led to the development of methods employing concentrated sodium chloride solutions for the isolation of anti-polysaccharide antibodies from pneumococcal precipitates or agglutinates (Heidelberger & Kendall, 1936; Heidelberger & Kabat, 1938). However, attempts to isolate anti-protein antibodies by using this salt were unsuccessful (Oudin & Grabar, 1944).

Kleinschmidt & Boyer (1952*a,b*) reported that the formation of protein-antibody specific immune precipitates was inhibited by concentrated solutions of certain electrolytes, by detergents, denaturing agents and other substances of low molecular weight. However, only recently have electrolytes such as sodium thiocyanate (Dandliker *et al.* 1967), sodium iodide (Avrameas & Ternynck, 1967) and magnesium chloride (Avrameas & Ternynck, 1969) been used for the elution of antibodies from immunosorbents.

The purpose of the present work was to study the action of various electrolytes on specific immune precipitates and on complexes formed between anti-

bodies and their homologous insolubilized antigens.

### MATERIALS AND METHODS

*Materials.* Crystalline human, bovine and rabbit serum albumin, and human, rabbit and sheep IgG,\* were purchased from Pentex Inc., Kankakee, Ill., U.S.A. Horseradish peroxidase (RZ3 and RZ0.6, RZ denoting the extinction ratio of peroxidase at 403 nm versus 278 nm and indicating the degree of purity) was obtained from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany. The salts used were of analytical grade where possible, or of the highest purity available.

*Antisera.* The rabbit antisera to peroxidase and to human albumin and IgG were prepared by immunization procedures described by Avrameas & Ternynck (1969).

Sheep anti-(rabbit IgG) and anti-peroxidase sera were prepared as described by Avrameas, Taudou & Ternynck (1971). The animals were bled 4, 5, 7 and 9 months after the first antigen administration. Relative avidities of the above antisera were estimated by using an immunosorbent technique. Standard quantities of rabbit IgG or peroxidase immunosorbent were incubated with increasing concentrations of the antisera and the amounts of antibodies absorbed were plotted versus the concentration of the antisera. The slopes of the curves were taken to represent the relative avidities of the antisera. It was

\* Abbreviation: IgG, immunoglobulin G.

found that the avidity increased with time after the first antigen administration.

Horse antiserum to whole rabbit serum was obtained from the Institut Pasteur, Paris, France. Horse anti-(pneumococcal polysaccharide T) serum and pneumococcal polysaccharide T were kindly given by Dr A. M. Staub of the Institut Pasteur.

**Protein determination.** The concentration of protein was determined by a modified biuret method (Kabat, 1961) with rabbit IgG as standard. Peroxidase content of different preparations was estimated from the  $E_{403}^{1\%}$  ( $E_{1\text{cm}}^{1\%} = 22$ ).

**Immuno-electrophoretic analysis.** Immuno-electrophoresis was performed by the method of Grabar & Williams (1953), but with gels of agarose instead of agar.

**Double diffusion.** This was performed by the procedure of Ouchterlony (1949).

**Antibody determination.** Antibody present in different preparations was determined by a quantitative precipitation test (Heidelberger & Kendall, 1935).

**Preparation of immunosorbents.** The immunosorbents were prepared by co-polymerization at pH 5 of four parts of bovine serum albumin and one part of the corresponding antigen with glutaraldehyde as the cross-linking agent, by a procedure described by Avrameas & Ternynck (1969).

**Experimental procedures used with immunosorbent-antibody complexes.** To measure the extent of action of electrolytes or distilled water on the complex formed between an antibody and its homologous insolubilized

antigen, the immunosorbents were employed in a large excess (50 mg of insolubilized antigen) compared with the quantity of antibody (10 mg) to be adsorbed. Experiments were performed by either the batchwise or the column procedure (Avrameas & Ternynck, 1969).

**Experimental procedure used with antigen-antibody specific precipitates.** Antibodies were precipitated from their respective antisera by the homologous antigen in the equivalence zone and in antibody and in antigen excess. The specific precipitates formed were then treated as described by Avrameas & Ternynck (1967). The following antigen-antibody systems were assayed: human serum albumin-rabbit anti-albumin, human IgG-rabbit anti-IgG, rabbit IgG-sheep anti-IgG, peroxidase-sheep anti-peroxidase, pneumococcal polysaccharide T-rabbit or horse anti-(pneumococcal polysaccharide T), and bovine trypsinogen-rabbit anti-trypsinogen.

**Exclusion chromatography.** Columns (100 cm  $\times$  2.5 cm) of Sephadex G-200 or G-100 that had been equilibrated for 48 h with the electrolyte solution were used at room temperature. Each chromatographic peak was tested by double diffusion and immuno-electrophoresis.

## RESULTS

**Effect of electrolytes on immunosorbent-antibody complexes.** Adsorbed antibody could be fractionated into different populations by using increasing

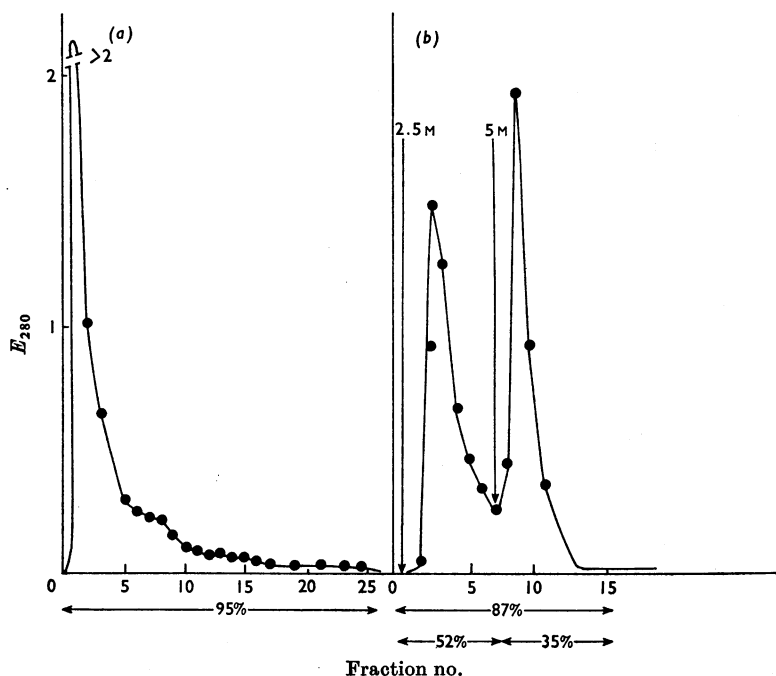


Fig. 1. Elution at 4°C of human serum albumin antibodies fixed on the homologous immunosorbent packed in a chromatographic column (2 cm  $\times$  10 cm); 23 mg of antibodies was adsorbed. (a) Antibodies were eluted with glycine-HCl buffer, 1.0.2, pH 2.8 (22 mg). (b) Antibodies were eluted with 2.5 M-MgCl<sub>2</sub> (12 mg), then (↓) 5 M-MgCl<sub>2</sub> (8 mg). Fractions (3 ml) were collected. The flow rate was 20–25 ml/h.

concentrations of electrolytes (Fig. 1). At high electrolyte concentration neither the ions used nor the pH of the solution affected the yield of antibody to a significant degree (Table 1). The elution of antibody was earlier at 20°C than at 4°C (Fig. 2) whether glycine-HCl buffer or an electrolyte solution was used, and a given volume of electrolyte could elute only a certain amount of antibody.

Distilled water may be used for the elution of antibodies fixed on immunosorbent (Fig. 3). About 50% of the fixed antibodies could be eluted with certain of the antigen-antibody systems assayed, namely human IgG-rabbit anti-IgG, rabbit IgG-sheep anti-IgG and human serum albumin-rabbit anti-albumin.

*Effect of electrolytes on specific immune precipitates.* The results presented in Table 2 were obtained

Table 1. *Elution by different electrolyte solutions of antibodies fixed on an immunosorbent*

Human serum albumin antibodies (23 mg) prepared in rabbits were adsorbed on the immunosorbent. Elution was performed with 2 × 10 ml of 2.5 M solution followed by 1 × 10 ml of 5 M solution.

Eluting solution	pH of the solution	Quantity of antibody isolated (mg)
NaSCN	7.0	17.5
NH <sub>4</sub> I	9.0	17.7
LiI	9.0	18.4
NaI	9.0	19.0
KI	9.0	17.7
MgCl <sub>2</sub>	7.0	19.0
Glycine-HCl buffer	2.8	19.7

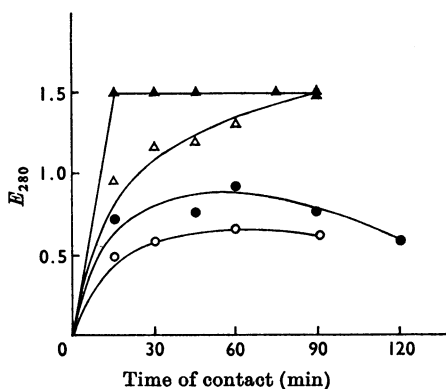


Fig. 2. Elution of human serum albumin antibodies fixed on the homologous immunosorbent shown as a function of the elution temperature and the time of contact with the eluent. Elution was carried out with glycine-HCl buffer, 1.0.2, pH 2.8, at 4°C (Δ) and at 20°C (▲) and with 2.5 M-MgCl<sub>2</sub> at 4°C (○) and at 20°C (●).

with the human serum albumin-rabbit anti-albumin system, but similar results were also recorded with human IgG-rabbit anti-IgG, rabbit IgG-sheep anti-IgG, peroxidase-sheep anti-peroxidase, pneumococcal polysaccharide T with antibodies to pneumococcal polysaccharide T prepared in rabbit or horse, and trypsinogen-

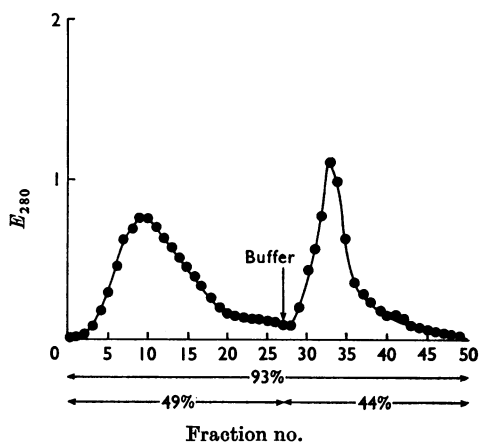


Fig. 3. Elution at 4°C of human IgG antibodies fixed on the homologous immunosorbent packed in a chromatographic column (2.5 cm × 7 cm); 82 mg of antibodies was adsorbed. Antibodies were first eluted with distilled water (40 mg) then (↓) glycine-HCl buffer, 1.0.2, pH 2.8 (36.5 mg). Fractions (6 ml) were collected. The flow rate was 20-30 ml/h.

Table 2. *Dissolution of specific precipitates in solutions of bivalent cations*

The electrolyte solution (3 ml) was added to 3 mg of human albumin-rabbit anti-(human albumin) precipitate. For experimental details see the text.

Concn. of salt ... Salt (pH 6.8-7.2)	Quantity of precipitate dissolved (mg)			
	0.5 M	1 M	2 M	3.5 M
MgI <sub>2</sub>	2.22	2.80	3.00	3.00
CaI <sub>2</sub>	2.10	2.75	2.80	3.00
SrI <sub>2</sub>	1.70	2.80	2.80	3.00
BaI <sub>2</sub>	2.13	2.80	2.80	3.00
MgBr <sub>2</sub>	0.81	1.86	3.00	3.00
CaBr <sub>2</sub>	0.48	1.05	2.80	3.00
SrBr <sub>2</sub>	0.48	0.99	2.76	3.00
BaBr <sub>2</sub>	0.63	1.41	2.76	3.00
MgCl <sub>2</sub>	1.14	1.40	3.00	3.00
CaCl <sub>2</sub>	0.55	1.05	2.04	3.00
SrCl <sub>2</sub>	0.68	0.68	1.40	1.53
BaCl <sub>2</sub>	1.70	2.50	3.00	—

rabbit anti-trypsinogen systems. Clearly bivalent cations are more effective than univalent cations (cf., e.g., Table 1 of Avrameas & Ternynck, 1967) and that their dissolving power is as follows:  $Mg^{2+} > Ba^{2+} > Ca^{2+} > Sr^{2+}$  (Table 2).

A given volume of electrolyte dissolved only a certain amount of precipitate and solubilization of precipitates by electrolytes seemed not to be time-dependent. Further, the logarithm of the solubility of the precipitate appears to be directly proportional to the molarity of the electrolyte.

Specific precipitates obtained in antigen excess were more effectively dissolved than precipitates formed at equivalence, whereas precipitates obtained in antibody excess were less readily dissolved (Fig. 4). It would also appear that the quantity of dissolved precipitate depends on the nature of the antigen and antibody present in the specific precipitate. Thus, for example, a precipitate formed at equivalence by pneumococcal polysaccharide T and rabbit anti-(pneumococcal polysaccharide T) serum was 60% less soluble in 2.5M-magnesium chloride than a precipitate formed at equivalence by albumin and rabbit anti-albumin serum.

Electrolytes such as 2.5M-magnesium chloride or 2M-sodium iodide dissolved larger quantities of proteins from precipitates formed by antibodies of low avidity than from precipitates formed by antibodies of high avidity. Thus in 2.5M-magnesium chloride 83, 67, 55 and 50% of the protein was

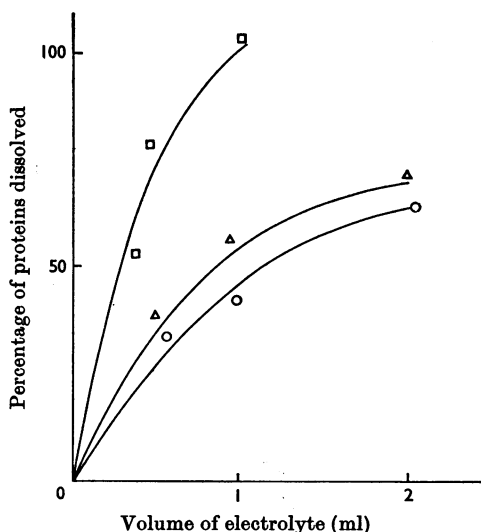


Fig. 4. Solubility of an albumin-anti-albumin precipitate in 2.5M-MgCl<sub>2</sub>, in relation to the antigen-antibody molar ratio in the precipitate: O, antibody excess (molar ratio, 1:6); Δ, equivalence zone (molar ratio, 1:3); □, antigen excess (molar ratio, 1:2).

dissolved from precipitates formed by rabbit IgG and sheep anti-IgG sera taken respectively 4, 5, 7 and 9 months after the first antigen administration (see the Materials and Methods section). Similar results were obtained with antisera from sheep immunized with peroxidase.

Exclusion chromatography of a mixture of normal rabbit IgG and peroxidase on Sephadex G-200 equilibrated with 2.5M-magnesium chloride gave two chromatographic peaks, the first containing IgG and the second peroxidase (Fig. 5a). Exclusion chromatography on the same column of peroxidase-rabbit anti-peroxidase specific precipitate, formed in the equivalence zone and dissolved in 2.5M-magnesium chloride, also gave two chromatographic peaks (Fig. 5b). The first contained peroxidase and was eluted before the IgG peak of the reference column. The second peak was free of peroxidase and was located in the same position as the rabbit IgG of the reference column. Tubes corresponding to the two chromatographic peaks were pooled, dialysed against 0.85% sodium chloride and concentrated. The first fraction formed a heavy precipitate. After centrifugation the supernatant was tested by double diffusion and immunoelectrophoresis and was found to be free of peroxidase or rabbit IgG. No precipitation was noted with the second chromatographic peak, which when tested by double diffusion and immunoelectrophoresis was found to contain rabbit anti-peroxidase antibody but not free or bound peroxidase. Peroxidase and specifically purified rabbit anti-peroxidase antibody, in the ratio found at the equivalence point, were dissolved separately in 2.5M-magnesium chloride, mixed and then passed through the same Sephadex G-200 column described above (Fig. 5c). The chromatographic profile was similar to that obtained with the dissolved peroxidase-anti-peroxidase precipitate (Fig. 5b) and the same constituents were found in the two chromatographic peaks. Similar results were obtained with peroxidase-anti-peroxidase precipitates that were formed in antibody or antigen excess, and with pneumococcal polysaccharide T-rabbit anti-(pneumococcal polysaccharide T) precipitates (Fig. 5d), in the equivalence zone or in antibody or antigen excess. The results were similar whether 2.5M-magnesium chloride or 2M-sodium iodide was used. Chromatographic columns equilibrated with high concentrations (above 3M) of magnesium chloride or sodium iodide were not effective because the beads of Sephadex G-200 shrank and lost their usual physical properties, causing occlusion.

Exclusion chromatography of a mixture of normal sheep IgG and peroxidase on a column of Sephadex G-100 equilibrated with 2.5M-magnesium chloride or 2M-sodium iodide gave two chromatographic

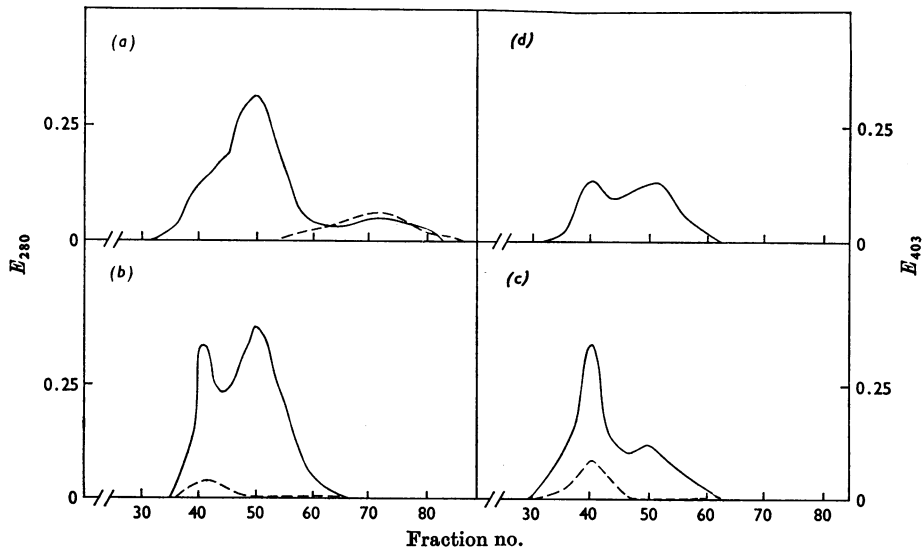


Fig. 5. Exclusion chromatography on Sephadex G-200 equilibrated with 2.5M-MgCl<sub>2</sub> of: (a) a mixture in 2.5M-MgCl<sub>2</sub> of normal rabbit IgG (12 mg) and peroxidase (6 mg); (b) peroxidase (6 mg)-rabbit anti-peroxidase (12 mg) specific precipitate treated with 2.5M-MgCl<sub>2</sub>; (c) a mixture in 2.5M-MgCl<sub>2</sub> of peroxidase (6 mg) and pure anti-peroxidase antibodies (12 mg); (d) pneumococcal polysaccharide T (1 mg)-rabbit anti-(pneumococcal polysaccharide T) (18 mg) specific precipitate treated with 2.5M-MgCl<sub>2</sub>. —,  $E_{280}$ ; ----,  $E_{403}$ .

peaks. The first, emerging with the void volume of the column, contained sheep IgG and the second contained peroxidase. Chromatography of peroxidase-sheep anti-peroxidase precipitate dissolved in 2.5M-magnesium chloride or 2M-sodium iodide on the same column gave only one chromatographic peak emerging with the void volume of the column. When the specific precipitate was dissolved in 5M-sodium iodide and passed through a column equilibrated with the same concentration of sodium iodide, two chromatographic peaks were obtained. The first, emerging with the void volume, contained peroxidase and sheep anti-peroxidase antibody and the second contained only free peroxidase.

## DISCUSSION

The quantity of proteins dissolved by a given electrolyte from an immune precipitate depends on the nature of the antigen-antibody system examined and on the proportion of the antigen in the precipitate. Further, for a given immune precipitate the quantity of proteins dissolved is a function of the temperature, the pH, the volume of solution used and the molarity of the electrolyte. The results indicate that, for the same temperature, volume and pH, the logarithm of the solubility of the precipitate is directly proportional to the molarity of the electrolyte. All the above

statements are in good agreement with the relationship established by Cohn & Ferry (1943) for the solubility of a protein in a solution of electrolyte. A specific precipitate might then be considered in the same way as a given protein and its solubility, as with any other protein, might depend on the repartition and proportion of its corresponding polar (hydrophilic) and apolar (hydrophobic) groups (Cohn & Ferry, 1943).

Only antigen-antibody complexes and free antibodies were detected when the precipitates were dissolved in solutions of electrolytes of low ionic strength (e.g. 2.5M-magnesium chloride or 2M-sodium iodide). It seems that dissociation of the antigen from antibody occurs and that this dissociation depends on the ionic strength of the electrolyte. It could be argued that only some of the antibody molecules were dissociated from the specific precipitate and that the rest of the antigen-antibody complex remained unchanged. However, the results obtained with pure antibodies and antigen that were separately dissolved in the same electrolyte and then allowed to react do not support this hypothesis, because in the final reaction mixture free antibody and antigen-antibody complexes were found. All the above evidence seems to indicate that the association constant of the antigen-antibody reaction was modified, being maximum under physiological conditions, decreasing

with increasing concentrations of electrolytes, and becoming virtually zero in saturated solutions of electrolytes. This hypothesis is in good agreement with that formulated by Heidelberger and collaborators (Heidelberger *et al.* 1936) to explain the dissociation by a 15% solution of sodium chloride of anti-polysaccharide antibodies from specific precipitates.

The experiments performed in the present work with antisera containing antibodies of different avidities indicate a close relationship between affinity and solubilization-dissociation. Kleczkowski (1965), by using distilled water, dissolved a specific albumin-rabbit anti-albumin precipitate that was prepared with a serum taken 3 weeks after immunization; with the anti-albumin antisera utilized in the present study, which were obtained from hyperimmunized animals, we were unable to dissolve the precipitate with distilled water. However, when the same antibodies were adsorbed on an albumin immunosorbent 50% of the antibodies were eluted with distilled water. It seems probable, therefore, that the affinity and consequently the association constant between an antibody and antigen is appreciably decreased when the antigen is insolubilized on an immunosorbent. This phenomenon might also explain why the nature of the eluting ions does not appreciably affect the yield of antibody from immunosorbents, whereas the extent of solubilization of an immune precipitate is largely dependent on the nature of the ions employed.

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