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Note

Demonstration of soluble immune complexes by analytical isotachophoresis

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The basic principle of isotachophoresis whereby ions are separated on the basis of net mobility has been known for many years and been described by several authors [1, 2]. However, it was only in 1970 that Arlinger and Routs [3] and Svendsen and Rose [4] applied the technique to the fractionation of protein mixtures. The present report is the first to describe detection of soluble immune complexes by means of analytical isotachophoresis. Advantages of the technique include rapidity of analysis, i.e., approximately 30-min running time, and small sample size, as little as 2—10 μl of sample containing microgram concentrations of protein.

EXPERIMENTAL

Chemicals

Tris (hydroxymethyl) aminomethane (tris), $Ba(OH)_2$ and HCl were obtained from Fisher Scientific (Pittsburgh, Pa., U.S.A.). Insolubilized protein A (protein A Sepharose CL-4B) was obtained from Pharmacia (Uppsala, Sweden). 2-Amino-2-methyl 1,3-propanediol (ammediol) was purchased from Eastman Kodak (Rochester, N.Y., U.S.A.). Hydroxypropylmethycellulose methyocel A 4M premium was a gift of Dow Chemical (Midland, Mich., U.S.A.).

Methods

A standard quantitative precipitin test similar to that of Heidelberger and Kendall [5] was performed. Rabbit immunoglobulin G (IgG) was prepared by passing rabbit antiserum to bovine serum albumin (BSA) over insolubilized

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protein A which binds to IgG through its F(c) portion and then eluting the absorbed IgG with 0.02 *M* glycine—HCl, pH 2.5. After the pH of IgG was readjusted to 7.3, increasing microgram amounts of BSA were mixed with a fixed concentration of rabbit anti-BSA IgG. The antibody—antigen mixtures were incubated at 37° for 1 h and at 4° for 6 days. The incubation tubes were then centrifuged at 3000 *g* for 15 min and the precipitates and supernatants were collected for study.

Precipitates were washed three times with cold 0.05 *M* phosphate buffered saline (PBS), pH 7.3, and the pellets were redissolved in 0.1 *N* sodium hydroxide for spectrophotometric examination at 280 nm. Identical mixtures of BSA and rabbit IgG, which did not contain antibodies to BSA were incubated together and processed in the same fashion. These served as the negative controls in the isotachophoretic analysis of the supernatants above the precipitated immune complexes.

Analytical isotachophoresis studies were performed with an LKB 2127 Tachophor apparatus. The separation chamber consisted of a 23-cm PTFE capillary tube (0.5 mm I.D.) maintained at a constant temperature of 20°. The apparatus was equipped with a thermal detector and with a UV detector set at 280 nm. The leading electrolyte, 0.005 *M* HCl—ammediol, pH 8.5 was supplemented with 0.04% A-4 methyl cellulose (Dow Chemical). The terminal buffer was 0.01 *M* ϵ-amino caproic acid (EACA) adjusted to pH 9.5 with $Ba(OH)_2$. A 0.01% solution of ampholine pH 3.5—10 (LKB) was used as a non-UV absorbing spacer. Analyses of the individual supernatants were performed at a constant current of 75 μA; the voltage increased from 3 to 20 kV during the running time of approx. 25 min per sample.

RESULTS AND DISCUSSION

A graph plotting optical density of the precipitate against the amount of antigen added is shown in Fig. 1. The equivalence point was established be-

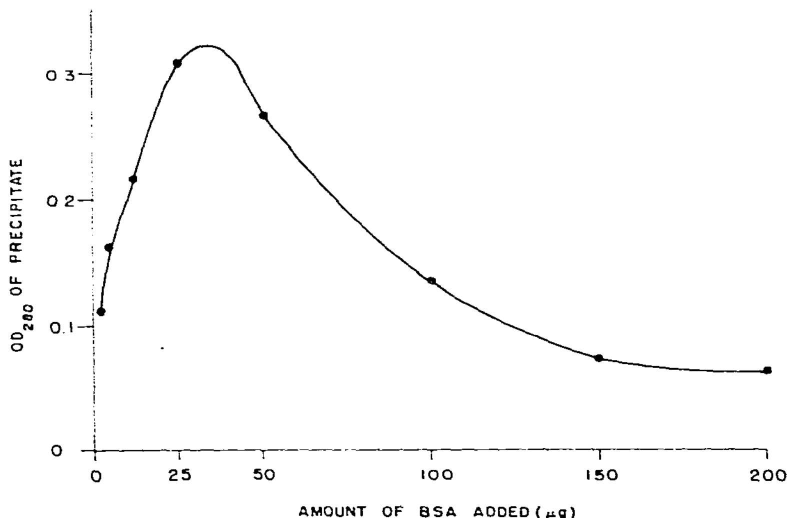

Fig. 1. Precipitin curve of bovine serum albumin (BSA) with IgG anti-BSA.

tween 30 and 35 μg of BSA. Supernatant material taken from the area of marked antigen excess provided a source of soluble immune complexes [5].

In those situations where rabbit IgG without antibody activity to BSA was mixed with increasing concentrations of BSA (Fig. 2, 1—5), analytical isotachophoresis demonstrated in these negatives controls an unchanging level of IgG as denoted by the areas under the black bar, and a progressive increase in the concentration of BSA, noted by the area under the open bar. In contrast in the isotachophoretic analyses shown in Fig. 2, 6—10, of the supernatants above the immune complex precipitates it can be seen that at point 7 of Fig. 2 there has been a reduction in the total amount of IgG. At point 8 in Fig. 2 a soluble complex appears as noted by the striped bar. The size of this complex increases as antigen excess increases and this is most evident in point 10, Fig. 2.

In Fig. 3 (1) the striped area represents the bulk of the IgG. Fig. 3 (2) shows the supernatant at equivalence; the total amount of IgG is reduced following its loss in the insoluble precipitate and a soluble immune complex appears with a net mobility that is intermediate between that of IgG and BSA. Passage of supernatant material over insolubilized protein A removed both the original IgG peak and the middle peak which as a soluble immune complex would also contain IgG. This is shown in Fig. 3 (3).

Serological activity of samples analyzed by isotachophoresis is presented in Table I and supports the hypothesis that the newly formed intermediary peak was indeed an immune complex. Samples containing soluble immune complexes had complement fixing activity that could be removed by absorption with protein A confirming the presence of IgG-containing components. A similar mixture of BSA and rabbit IgG which did not have anti-BSA specificity did not result in the formation of an immune complex as measured by complement fixing activity or isotachophoretic analysis (see Fig. 2, 2—5).

In summary the combination of increasing amounts of antigen with a fixed amount of homologous antibody results in the formation of both insoluble and soluble immune complexes. If one studies the supernantants above the removed insoluble immune complexes soluble complexes can be identified by complement fixing activities and by the formation of a unique intermediate peak identified by means of analytical isotachophoresis. If one adsorbs the IgG components from the supernatants by means of protein A both complement fixing activities and the appropriate IgG and IgG-containing components seen by isotachophoresis are removed. Neither complement fixing activity nor inter-

Fig. 2. Isotachopherograms of supernatants obtained from various points of the precipitin curve in Fig. 1. 1—5 represent the preimmunization rabbit IgG controls; 6—10 represent the points to which 0, 12.5, 25, 100 and 200 μg of BSA were added to a fixed concentration of antibody. Solid black bar, IgG; open bar, free BSA and striped bar, immune complex. A 5-μl volume of each sample was injected with 1 μl of a 0.01% solution of ampholine, pH 3.5—10. A = increasing UV absorption; R = increasing resistance; t = time.

Fig. 3. Isotachopherograms of supernatants obtained from various points of precipitin curves. 1 = IgG alone; 2 = sample of supernatant taken at a point where the amount of antigen was in excess of the amount of antibody directed against it. This shows a reduction in the free IgG and the formation of a soluble immune complex (IC); 3 = sample of supernatant identical to 2 after absorption on protein A, IgG and IgG-containing complexes have been removed. A 5-μl volume of each sample was injected with 1 μl of a 0.01% solution of ampholine, pH 3.5—10. A = increasing UV absorption; R = increasing resistance; t = time.

1 6

2 7

3 8

4 9

5 10

A

R

t

30 SEC

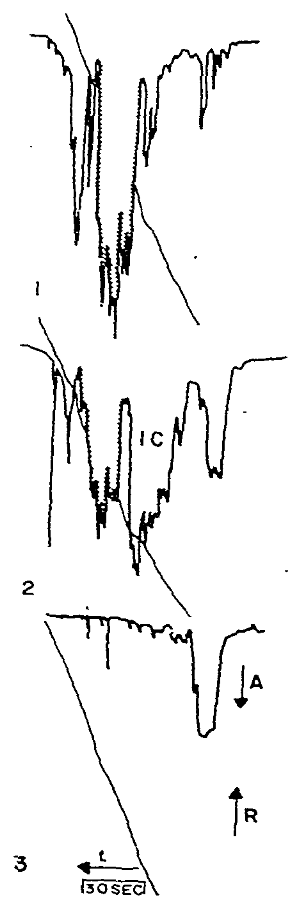

TABLE I

COMPLEMENT FIXING ABILITY OF SOLUBLE SUPERNATANT SPECIMENS

Supernatant specimen*	BSA added (μg)	Complement fixation titer
6	0	0
7	12.5	0
8	25	0
9	100	1:16
10	200	1:32
10**	200	0

*Specimens correspond to those described in Fig. 2.

**Supernatant after adsorption of protein A (corresponding to Fig. 3 (3).

mediate peaks are demonstrated by simply mixing BSA and IgG without BSA antibody specificity.

This is the first reported demonstration of the identification of soluble immune complexes by means of analytical isotachophoresis. It is hoped that the technique can be extended to the study of a wide variety of antigen and antibody systems to exploit the speed and convenience allowed by analytical isotachophoresis.

REFERENCES

1 L. Arlinger, Protides Biol. Fluids, Proc. Colloq., 22 (1974) 661.
2 F.M. Everaerts, M. Geurts, F.E.P. Mikkers and Th.P.E.M. Verheggen, J. Chromatogr., 119 (1976) 129.
3 L. Arlinger and R.J. Routs, Sci. Tools, 17 (1970) 21.
4 P.J. Svendsen and C. Rose, Sci. Tools, 17 (1970) 13.
5 M. Heidelberger and F.E. Kendall, J. Exp. Med., 62 (1935) 697.