Radioautographic Analysis of Soluble Antigen-Antibody Complexes Separable by Paper Electrophoresis. (25760)

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The biological importance of soluble antigen-antibody complexes has been previously well established by inducing cutaneous reactions(1,2), anaphylaxis(2,3), and serum sickness(3,4), with soluble complexes prepared *in vitro*. The physical-chemical properties of such complexes have been studied by several technics including free boundary electrophoresis(5,6,7). Use of paper electrophoresis in analysis of soluble complexes, prepared for *in vivo* studies suggests itself, but its value is limited when conventional methods of staining are used in evaluating low concentrations of protein usually applied. In this study radioautographs of paper electrophoretic patterns containing radioactively labelled soluble complexes permitted a simple and sensitive method of analysis.

Materials and methods. Electrophoretically homogenous bovine serum albumin (BSA) was used for iodination and immunization procedures. Anti BSA sera were prepared by repeated injections of 250 mg of BSA mixed with Freund's adjuvant/injection for 6 week period. Fifty mg were injected into each of 5 portals, (intravenous, intramuscular, intraperitoneal, subcutaneous and intradermal), twice a week for 6 weeks. Rabbits were bled by cardiac puncture 5 days following last injection. Sera were pooled, frozen rapidly with alcohol and dry ice, then stored at −10°C. The γ globulin fraction was separated off as needed using ammonium sulphate method described by Cushing and Campbell(8). BSA was iodinated with one atom of iodine/molecule of protein according to the method of Talmage(9). Adequate specific activity was obtained by using .5 ml of solution containing 25 mg of BSA and 10 mc I^{131} with activity greater than 20 mc/ml, with .1 ml of .01 N KI carrier. The solution was dialyzed 2 days against water, then allowed to equilibrate with .86% NaCl. Free I^{131} was less than 2% on measurements of activity using 10% TCA protein precipitation. I^{131} γ activity was measured in a well-type detector coupled to conventional scaler. Background activity was 4.6 to 6 counts/second. Samples were counted for a sufficient time to keep statistical counting error below 1%.

Preparation and analysis of BSA-anti BSA complexes. BSA I^{131} was added to anti BSA γ globulin in proportions to give combination at point of equivalence. The mixture was allowed to stand at 27°C for 2 hours, then placed at 4°C for 24 hours. The precipitate was washed repeatedly and brought to a known volume with cold .86% NaCl. To insure adequate suspension of the precipitate, the mixture was agitated in a Ratheon sonic oscillator at 9 KC/sec for 100 seconds. Samples were set aside for future incubation with excess unlabelled antigen. Aliquots of samples were counted for radioactivity and nearly identical samples were selected for incubation with .5 cc of excess antigen at 2.5, 5, 10 and 20 times the equivalence zone. Insoluble antigen-antibody content represented by undissociated radioactive BSA corresponded to 82, 77, 73 and 69% respectively for 2.5, 5, 10 and 20 times the equivalence zone. Suspensions were incubated 6 hours at 4°C with constant agitation, then centrifuged repeatedly until activity in supernatants reached equilibrium. Similar .03 ml aliquots were run 20 hours at room temperature in Spinco tent type paper electrophoresis apparatus, model R - series D, using barbiturate buffer at pH 8.6, μ .075. After drying, radioautographs were prepared by tightly pressing Dupont medical 508 X-ray film against the papers for 48 hours. Film was developed in the usual way and autographs scanned in a Bender and Hobein densiometer. Paper electrophoretic patterns of soluble complexes and of control sera were similarly stained and evaluated. Optical density of each band in the radioautographs was expressed as percentage concentration as indi-

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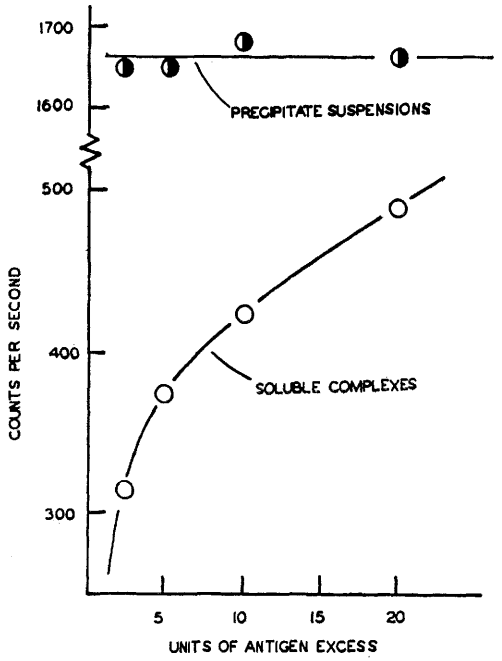

FIG. 1. Formation of soluble complexes is indicated by increasing radioactivity in supernatants with increasing amounts of antigen added in excess. At 20 × the equivalence zone approximately ½ of preformed labelled precipitate is dissolved in the form of soluble complexes, and aliquot measured contains 1 γ of BSA.

cated by the area under relevant parts of the tracing. Comparison with migration rates of normal rabbit serum proteins served to indicate relative electrophoretic mobilities of the soluble complexes. Control radioautographs of radioactive, electrophoretically homogenous BSA demonstrated 99% of isotopic activity located in albumin fraction.

Results. Micro-Kjeldahl analysis of labelled BSA indicated a specific activity of 470 counts/second for 1 γ of BSA I^{131}. Serial dilutions of labelled BSA, spotted on paper and radioautographed, revealed that concentrations of less than .1 γ of BSA were readily demonstrable in 48 hours. Aliquots of each supernatant, ranging in activity from 20-30 times this value, *i.e.*, containing 2-3 γ of soluble complexed BSA, dissolved in increasing antigen excess, were chosen for analysis (Fig. 1).

Radioautographs of the soluble complexes revealed 4 distinct bands (Fig. 2). Comparison of tracings of the autographs with tracings of control BSA and rabbit serum proteins demonstrated that the leading component migrated at the rate of normal BSA and that the remaining bands represented soluble complexes–one remaining at application point, and 2 migrating at the rates of β and α globulins respectively (Fig. 3). In previous work by Weigle *et al.* using BSA-rabbit anti BSA system, conventional methods of paper electrophoretic analysis revealed 3 bands. A band representing free BSA, and 2 bands migrating at rates of β and γ globulins were reported (10).

The most rapidly migrating complex is believed to be antigen rich on the basis of its migration rate and tendency of this fraction to increase in percentage concentration at the expense of the complex remaining at the application point, assumed to be antigen poor or antibody rich. The intervening band represents a complex of intermediate composition (Fig. 4). It is likely that the first 2 complexes represent the Ag2-Ab1 and Ag3-Ab2 complexes described by Singer(5,6,7), and that the varying weights and charges of the molecule account for their specific rates of electrophoretic migration. The fraction remaining at application point may be due to

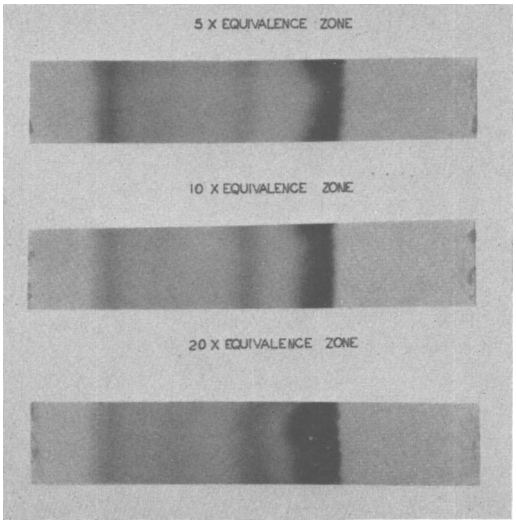

FIG. 2. Radioautographs of electrophoretic patterns of antigen-antibody complexes showing 3 bands: One remaining at application point, the other 2 migrating at rates of β and α serum globulins. Free BSA represented by the most rapidly moving fraction is almost completely absent in regions of low antigen excess with extensive washing of initial precipitate, but an excess is shown here for orientation.

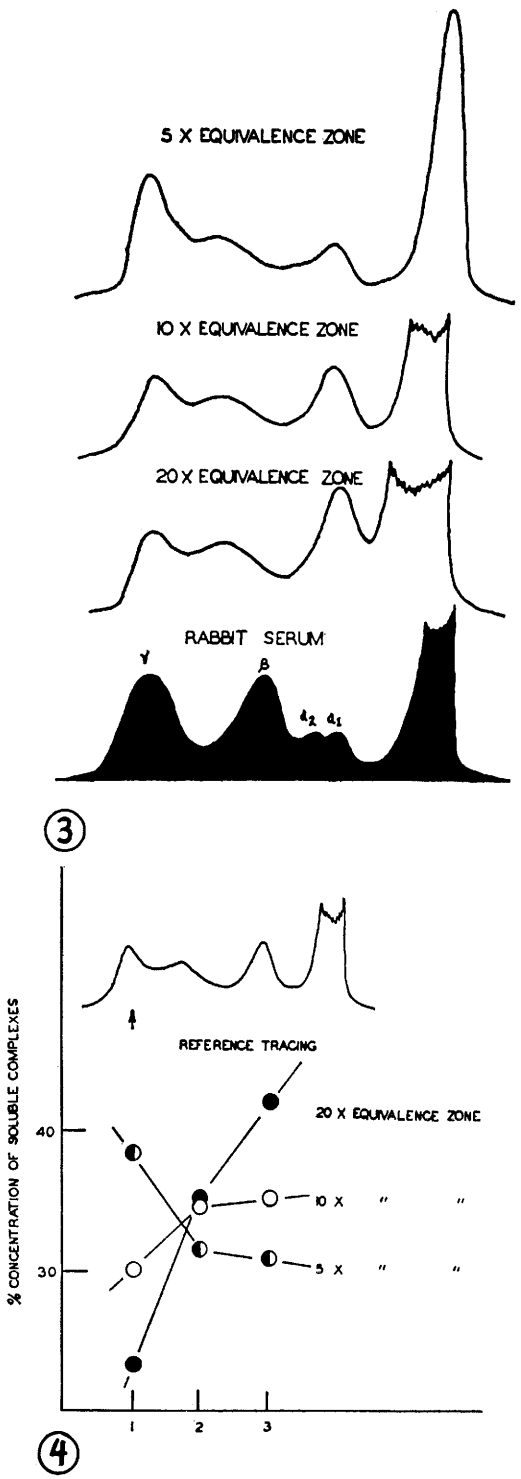

FIG. 3. Tracings of radioautographs of labeled soluble antigen-antibody complexes at 5, 10, and 20 × equivalence zone indicating their electrophoretic mobilities relative to a tracing of a stained paper electrophoretic pattern of control rabbit serum globulins. Leading fractions represent soluble complexes migrating at rate of α and β globulins, and the component remaining at application point may be reprecipitated complexes, or higher molecular weight complexes with a high affinity for the paper.

FIG. 4. Percentage concentration of each of the complexes at 5, 10, and 20 times the equivalence zone is indicated under the reference electrophoretic pattern for each of these complexes. The antigen-rich, fast migrating, complex increases in concentration at expense of the antigen-poor complex remaining at point of application. It would appear that the binding of BSA is divided equally between the 3 complexes in this system at a point somewhere between 5 and 10 times the equivalence zone.

a reprecipitation of insoluble aggregates as the system reequilibrated during time required for electrophoretic separation. Or the band may represent a soluble but higher molecular weight complex possessing a high affinity for paper supporting medium.

Summary. A radioautographic technic with a sensitivity many times greater than conventional staining methods is described for evaluation of paper electrophoretic studies of soluble antigen-antibody complexes. Electrophoretic mobilities of BSA-anti BSA soluble complexes relative to rabbit serum proteins are reported with a semi-quantitative analysis of percentage concentration for each of the complexes at various values of antigen excess.

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