ULTRA-MICRO PRECIPITIN PROCEDURE ON CELLULOSE ACETATE

A.S. Markowitz and Jon Isenberg

The Hektoen Institute for Medical Research of the Cook County Hospital, Chicago 12, Illinois

Received April 26, 1963

With the increased utilization of the immunochemical approach for the quantitation of substances present in biological fluids, it has become apparent that classical quantitative precipitin reactions have limited practical applicability due to the quantity of reactants consumed. Because of this reason, and others relating to necessary technical skill and speed in performing multiple determinations, a procedure was designed to obviate such objections.

Kohn (1) has shown that precipitin reactions can be performed on cellulose acetate and that the antigen-antibody complex can be suitably stained. Brackenridge (2) has shown that within limits a linear relationship obtains between dye uptake and protein concentration.

The following report concerns itself with a procedure which quantitates serum antibody as measured by the dye uptake of the immunologic precipitate on cellulose acetate.

Inasmuch as different proteins have different uptakes of dye, it was necessary to prepare a standard curve for each antigen-antibody system investigated. Since rabbit antisera was employed the standard curve was obtained by determining the dye binding capacity of a mixture of 10 parts of rabbit gamma globulin and 1 part of the antigen

under consideration. Protein concentration of the mixture was determined by micro-Kjeldahl and then adjusted to concentrations ranging from 0.029 micrograms nitrogen to 0.93 micrograms nitrogen for five lambda.

Cellulose acetate strips, 3 cm. x 5 cm., were wetted in veronol buffer, pH 8.6, u-0.07 and then blotted free of excess moisture. The strips were placed on a holder which is so designed as to suspend the major part of the strip (exclusive of the edges which rest on the holder) over an air space and increasing nitrogen concentrations of the protein mixture in a total volume of five lambda were then placed on individual strips with the aid of calibrated micropipets. After permitting diffusion of the protein through the strip they were placed in a 0.03% solution of nigrosin in 2% acetic acid for a period of from 9-12 hours. The strips were then rinsed in running tap water and blotted free of excess moisture with filter paper. The entire stained area was cut out and dissolved in 0.3 ml. of solvent (8.8 parts methylene chloride to 1.2 parts ethanol) with the aid of a glass stirring rod. The O.D. of approximately an 0.15 ml. aliquot of the above was determined with a micro cuvette attachment for the Coleman Junior Spectrophotometer at 545 mu.

Figure 1 shows the linear relationship obtained with this procedure employing a mixture of rabbit gamma globulin and human serum albumin. Such a curve was reduplicatable within 0.3% error.

As a rule, 12 dilutions of antigen were employed, all reactions conducted in duplicate. Ten lambda of antiserum was placed in the center of a previously prepared 3 x 5 cm

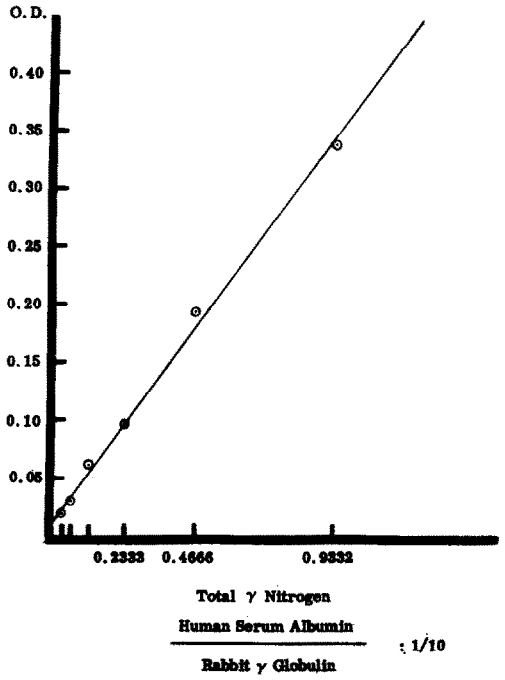


Figure 1.

strip as indicated above and permitted to diffuse through the cellulose acetate. Immediately thereafter, 5 lambda of the appropriate antigen concentration was superimposed upon the antiserum area. The strips were then placed under a layer of Whitmore Oil (Consolidated Labs, Chicago Heights, Illinois) and incubated at 37° C. for 14-18 hours. At the end of the incubation period, the strips were drained free of flowing oil and placed in a phosphate buffer, pH 8.2, elution bath equipped with a magnetic stirring device so as to keep the strips moving about. One and a half hours later the strips were removed and placed in a fresh phosphate buffer elution bath and kept for an equal period of time. This procedure was repeated for a total of six times and then stained as indicated above for the standard protein curves.

The precipitin lines which appeared as a darkly stained circle can be seen in Figure 2. Approximately 1 mm. on either side of the precipitin line was included when the circle was cut out and dissolved in the solvent. It was found that the best instrument for performing this was a 4 inch curved iris scissors.

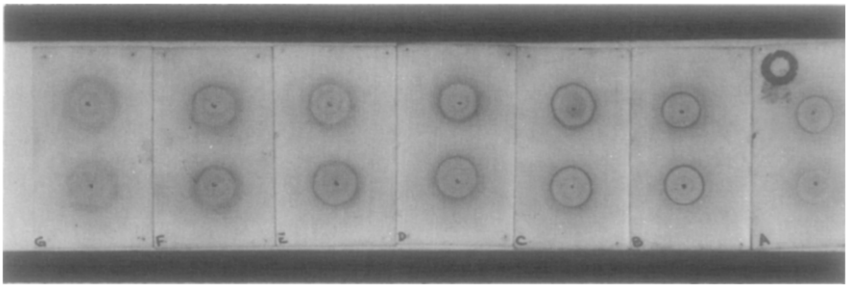


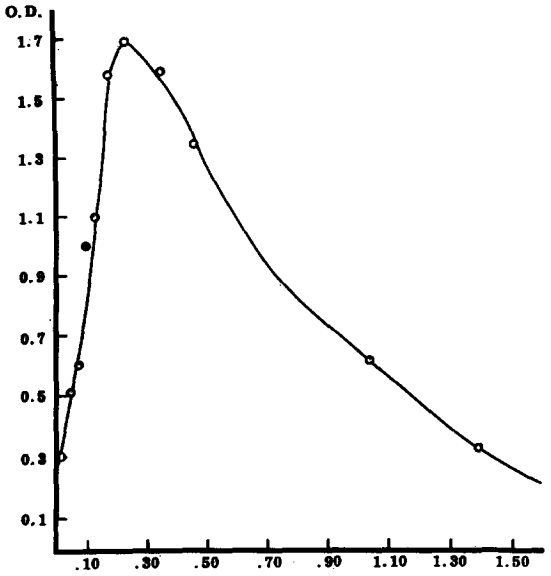
Figure 2

Nigrosin Stained Precipitin Reactions on Cellulose Acetate. A To G Represents Increments of Antigen Nitrogen Added

The antibody nitrogen content of two rabbit antisera, antihuman gamma globulin and anti-human serum albumin was determined by the quantitative precipitin procedure (3) and the nitrogen assayed by the micro-Kjeldahl method.

Figure 3 is representative of a plot of the data obtained for the HSA system. The curve presents O.D. plotted against micrograms antigen nitrogen added. It can be seen than an equivalence is reached, after which presumably antigen excess diminution of precipitate occurs with consequent drop in dye uptake. The antibody nitrogen was determined by extrapolation of the O.D. value at equivalence from the standard curve and subtracting the antigen nitrogen added from this figure.

With this procedure antibody nitrogen for the rabbit anti-HSA serum was found to be 130 micrograms N/ml. as



Human Serum Albumin – Rabbit Anti–Human Serum Albumin

compared to the value obtained by the Heidelberger procedure of 110 micrograms N/ml. Comparative values obtained for the HGG system were 103 micrograms N/ml. and 128 micrograms N/ml. respectively.

In any comparison on a quantitative basis of this method and that of the classical tube reaction, it must be kept in mind that the system described here is not a true three-dimensional one. While one cannot estimate the "blocking" effect of the cellulose acetate as the reactant molecules adsorb onto the surface of the fibers, it must be considered as contributory to negating the combining capacity of the reactants to some extent. With this in mind, it becomes apparent that absolute quantitative comparisons of the two methods is not feasible.

The merits of this method can be stated simply. It is exceedingly easy to perform, requires no special skill or

equipment, is highly reproducible for any given system (with 2-3%), and requires minimal amounts of reactants.

Its major demerit is that it cannot be truly quantitative in the sense that one can account for each molecule of antibody or antigen nitrogen. There are a number of technological precautions which must also be considered. The elution procedure if inadequately performed will leave residual protein to take up the dye and thus contribute to the values obtained. If care is not exercised in the cutting out of the precipitin circle erroneous values will be obtained. And lastly, it must be appreciated that at the low volume level this procedure operates, extrapolation of a value from 10 lambda to one milliliter can greatly magnify any existing initial error.

Keeping in mind all of the inherent drawbacks to this procedure, it nevertheless becomes evident that it represents a highly reproducible system and one which because of its relatively low consumption of reactants could lend itself to the immunoassay of many important biological materials.

References

1. Kohn, J., Nature, 180:986, 1957.
2. Brackenridge, C.J., Anal. Chem. 32:1353, 1960.
3. Heidelberger, M. and Kendall, F.E., J. Exper. Med., 62:697, 1935.