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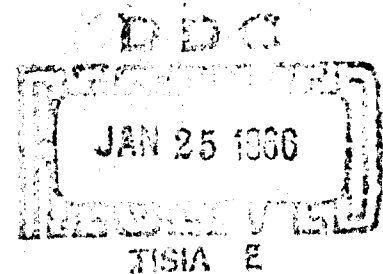
TECHNICAL MANUSCRIPT 273

DETERMINATION OF ANTIBODY
TO PURIFIED MICROBIAL ANTIGENS
BY AMMONIUM SULFATE COPRECIPITATION
(FARR TECHNIQUE)

Jack Gruber

George G. Wright

JANUARY 1966



UNITED STATES ARMY
BIOLOGICAL LABORATORIES
FORT DETRICK

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Fort Detrick, Frederick, Maryland

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DIRECTORATE OF MEDICAL RESEARCH

Project 1C014501B71A

January 1966

In conducting the research reported here, the investigators adhered to "Principles of Laboratory Animal Care" as established by the National Society for Medical Research.

ABSTRACT

The ammonium sulfate coprecipitation technique (ASCT) described by Farr in 1958 has been employed primarily with stable, well characterized antigens such as serum proteins. To facilitate study of the efficacy of immunization procedures and the role of serum antibody in resistance to infection, the application of ASCT to purified microbial antigens has been investigated. Studies with iodine 131-labeled protective antigen of Bacillus anthracis, using an equal volume of 2.6 M ammonium sulfate for coprecipitation, revealed differences in the combining capacities of various equine sera. Approximately 100% of a labeled protective antigen preparation was precipitated by a 1:20 dilution of hyperimmune pony serum, whereas approximately 20% was precipitated by a 1:20 dilution of normal horse serum. Similarly, using 3.2 M ammonium sulfate for coprecipitation, radioiodine-labeled purified enterotoxin B of Staphylococcus aureus revealed differences in the combining capacities of various rabbit sera for the enterotoxin antigen. At a final concentration of 1 microgram per milliliter, essentially 100% of the labeled toxin was precipitated by a 1:10 dilution of a pooled anti-enterotoxin serum, whereas only 10 to 20% of the toxin was precipitated by normal rabbit sera. With both systems, over a considerable range of dilutions of normal and immune sera, the proportion of antigen precipitated by immune sera remained significantly greater. Thus, the ASCT yields sensitive and quantitative measurements of antibody reactive with these microbial antigens.

DETERMINATION OF ANTIBODY TO PURIFIED MICROBIAL ANTIGENS
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The ammonium sulfate coprecipitation technique, first reported in detail in 1958 by Farr, provided a quantitative and sensitive assay of antibody to iodine 131-labeled bovine serum albumin.¹ Since that time, Farr and other workers have utilized this technique to investigate a variety of immunological phenomena, such as the production of antibody after X-radiation;² the cellular in vitro production of antibody following in vivo stimulation;³ the kinetics of the antigen-antibody reaction;⁴ and the types of antibody synthesized during the immune response.⁵ However, most investigators have employed in their studies the bovine serum albumin-antibovine serum albumin system, or have utilized for iodination and study other stable and well-characterized antigenic material as the serum proteins. For some time our concern has been with the development or application of serological procedures for sensitive and quantitative measurement of serum antibody to aid in testing the efficacy of vaccines and immunization procedures. We have been surprised to find that the ammonium sulfate coprecipitation technique of Farr has been virtually neglected by those studying resistance to infection. In 1962, Preter did employ the Farr technique to detect coproantibodies against Vibrio cholerae in humans immunized by the oral and parenteral routes.⁶ However, in his work he used relatively crude "O" antigen preparations obtained by cell fractionation, which probably contained numerous antigens. Intent on developing improved methods for the assessment of the efficacy of immunization, we have studied the applicability of the ammonium sulfate coprecipitation technique to systems utilizing purified antigenic material derived from microbial sources.

In this investigation we used two antigens derived from quite dissimilar bacteria. In the first instance we used the anthrax protective antigen prepared in our laboratory. This antigenic material is obtained from Bacillus anthracis strain V770-NP1-R grown anaerobically in a defined medium. The antigen is purified by chromatography of culture filtrates on DEAE cellulose columns and gel filtration. Immunoelectrophoresis has revealed the antigen to contain three components closely related serologically but differing in electrophoretic mobility. Preliminary ultracentrifuge study has indicated a molecular weight of the order of 80,000. This purified antigenic material combined with aluminum hydroxide suspension has been found to have considerable immunizing activity in guinea pigs. The second microbial antigenic material employed was staphylococcal enterotoxin B obtained from Staphylococcus aureus strain S6, and kindly supplied by Dr. E.J. Schantz of Fort Detrick. This material is purified by chromatography on Amberlite CG-50 resin and carboxymethyl cellulose. The enterotoxin B antigen has been characterized as a simple protein with a molecular weight of 35,000. It is a homogeneous material, as indicated by the analytical ultracentrifuge (2.89 g), free electrophoresis, and by agar gel diffusion tests. Its purity has been estimated as greater than 99%.

Iodination of the purified microbial antigens to obtain labeled material for serology was performed by the usual nitrous acid procedure or by a modified microdiffusion iodination technique.

A purified anthrax protective antigen preparation was labeled with iodine 131 by the nitrous acid method described by Johnson, et al.⁷ The effect of iodination on the precipitating activity of protective antigen was tested by Ouchterlony plate titration, which indicated full retention of that biological activity. Estimates based on values of 80,000 as the molecular weight and 0.1 milligram protein per milliliter of antigen indicated a labeling of approximately 3 atoms of iodine per molecule of antigen. Recovery of the labeled protective antigen from unbound iodine was obtained by passage through a Sephadex G25M gel column. Recovery of the protective antigen from the column, as determined by Ouchterlony precipitating activity, was coincidental with the elution of two radioactivity peaks obtained. This is indicated in Figure 1. Essentially all of the original added radioactivity could be recovered from the Sephadex columns. Usually, with this protective antigen preparation, 80 to 91% of the added biological activity was recovered, and the level of radioactivity present with the antigen fractions indicated an efficiency of labeling of 15 to 25% of the original radioactivity. Figure 1 illustrates a typical separation. In this instance we obtained 80% recovery of the biological activity and approximately 22% of the added radioactivity was present in the antigen peak material.

Since Farr's original technique depended on the solubility of uncombined labeled albumin in half-saturated ammonium sulfate, we next investigated the solubility of our labeled protective antigen at various concentrations of ammonium sulfate. Anticipating manipulations at various temperatures, we used molarity rather than per cent saturation in our work. Figure 2 indicates the solubility of the pooled labeled protective antigen at various concentrations of ammonium sulfate. The labeled antigen was insoluble at high ammonium sulfate concentrations, especially in the presence of normal serum, but at 2.8 M concentration, even with serum present, was relatively soluble. Approximately 70% of the labeled protective antigen, with normal serum present, was soluble in 2.8 M ammonium sulfate. To eliminate the possibility of some nonspecific precipitation of labeled antigen during serological tests we first treated our labeled preparation with 3.2 M ammonium sulfate. Then we tested the remaining soluble protective antigen for coprecipitation with normal and immune equine sera, using 2.8 M ammonium sulfate.

As Figure 3 indicates, we found marked differences in the combining capacities of various equine sera. Approximately 100% of the labeled protective antigen was precipitated by a 1:20 dilution of a hyperimmune pony serum, but only approximately 20% was precipitated by a 1:20 dilution of normal horse serum. As the antiserum was diluted, the proportion of antigen precipitated was decreased. Using precipitation of 50% of the radioactive antigen to characterize the serum, a direct relationship was noted between the concentration of antigen employed and the extent of coprecipitation. With a tenfold decrease in antigen concentration an increase in serum titer of approximately tenfold was observed.

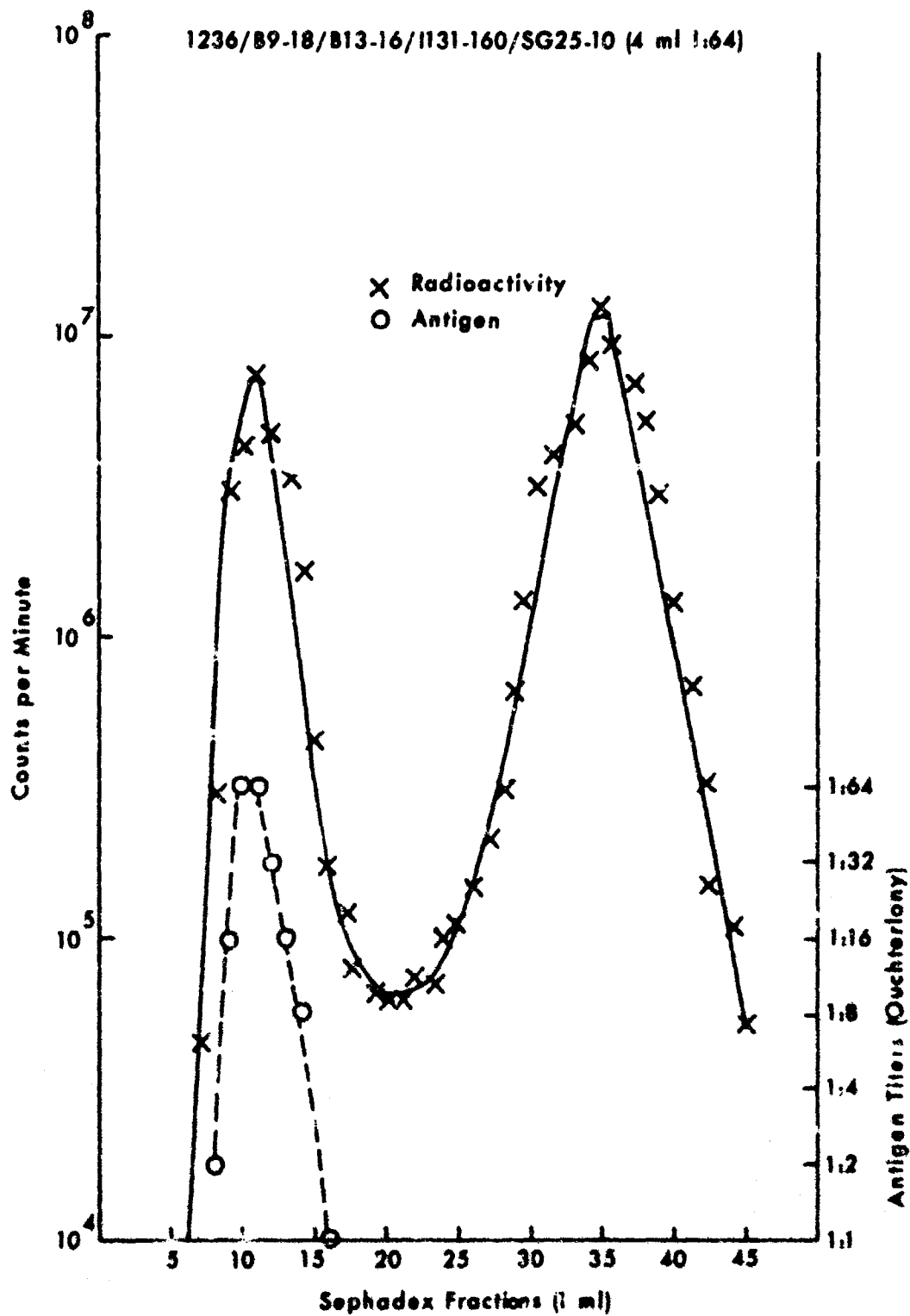


Figure 1. Recovery of Labeled Anthrax Protective Antigen by Sephadex Gel Filtration.

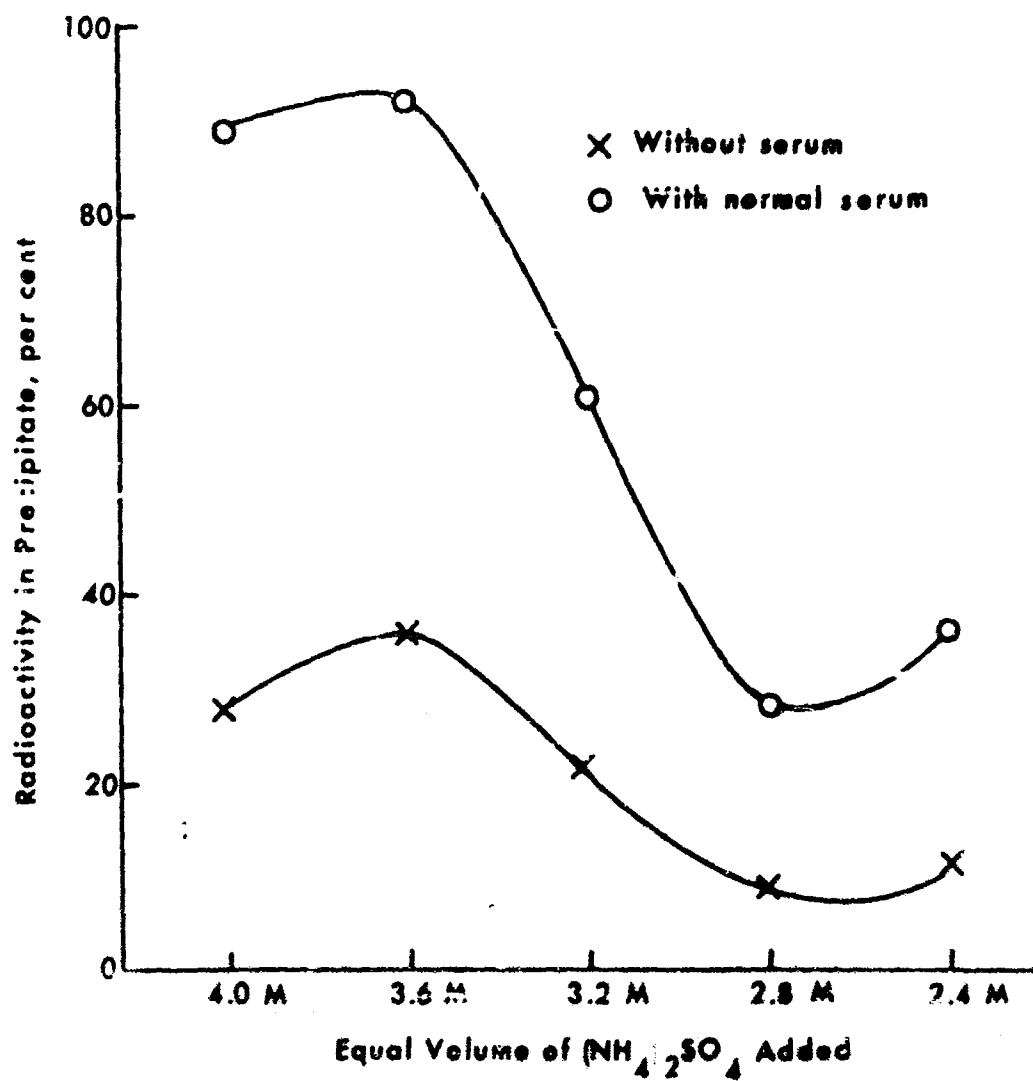


Figure 2. Solubility of Labeled Protective Antigen in Ammonium Sulfate.

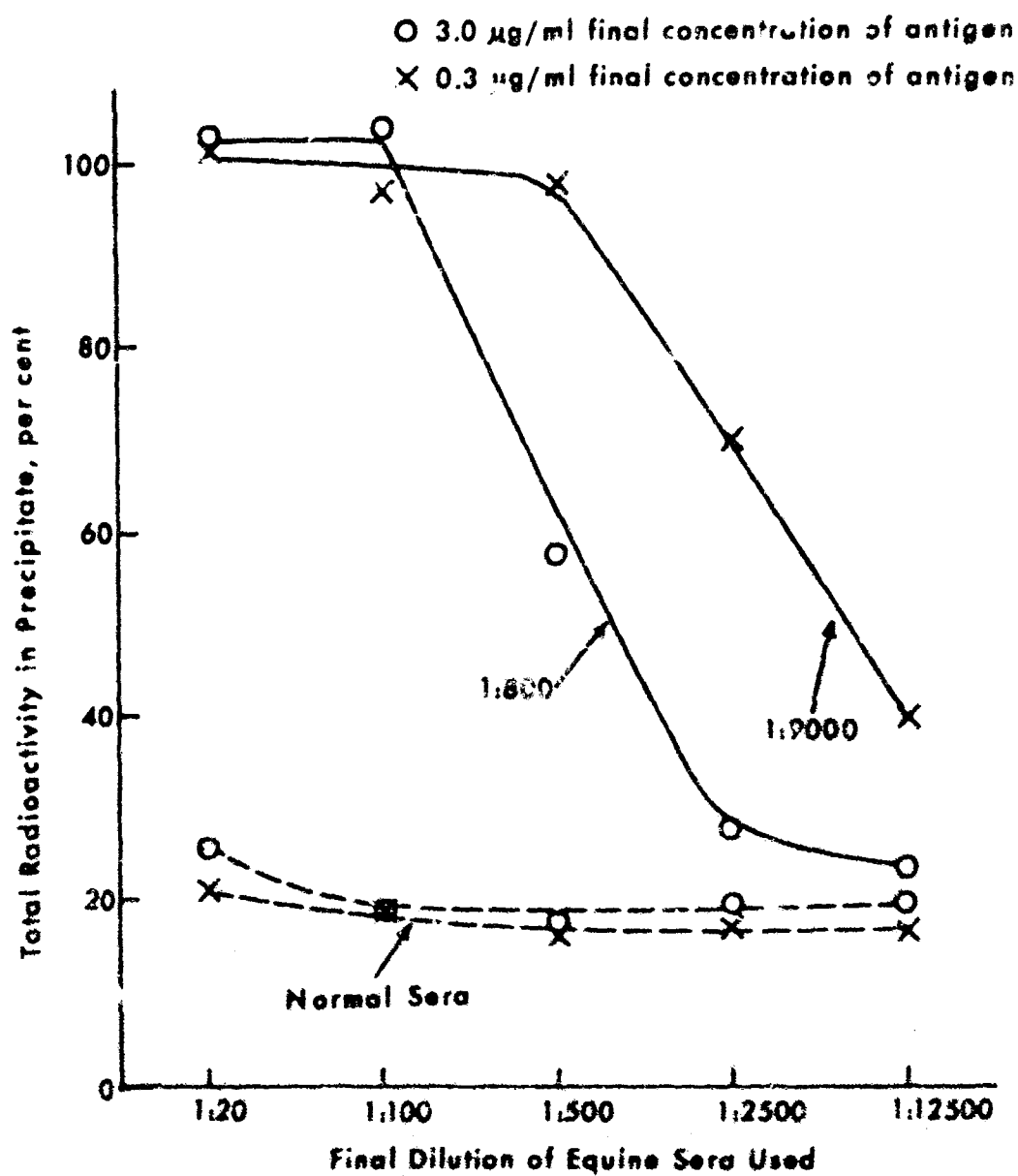


Figure 3. Coprecipitation of Labeled Protective Antigen and Equine Sera Using 2.3 M Ammonium Sulfate

We next investigated the applicability of using the Farr technique with an even more highly purified microbial antigenic material. Staphylococcal enterotoxin B was iodinated by a modification of a microdiffusion procedure of Banerjee and Ekins,⁸ which eliminates the addition of oxidizing agents to the protein and avoids dilution of its concentration. The labeled enterotoxin was separated from unbound iodine by dialysis. Calculations of the conversion of iodine-131 to the gaseous phase, of the diffusion of the iodine into the protein solution, and of the efficiency of labeling indicated that approximately 0.5 to 1.5 atoms of iodine were bound per molecule of toxin. Both the Ouchterlony and Oudin procedures indicated that essentially 100% of the precipitating activity of the enterotoxin was retained after iodination and dialysis.

For technical and manipulative reasons, when we started using the enterotoxin system for serology, we initiated the counting of the non-precipitated soluble radioactive antigen as an indication of the extent of coprecipitation. Therefore, the following graphs were plotted to indicate per cent solubility of the radioactive antigen in the presence of the test serum. As Figure 4 indicates, serological studies using 3.2 M ammonium sulfate for coprecipitation and radiolabeled enterotoxin B at a final concentration of 10 µg per ml revealed differences in the combining capacities of various rabbit sera for the enterotoxin antigen. Greater than 65% of the toxin antigen was precipitated by a 1:10 dilution of a pooled anti-enterotoxin serum; only an approximate 25% of the test antigen was precipitated by 1:10 diluted normal rabbit sera. Again, as in the anthrax system, as the antiserum was diluted, the proportion of toxin precipitated was decreased; at high dilution of immune sera, essentially none of the labeled toxin was precipitated.

Studies were also conducted using radiolabeled enterotoxin antigen at a final concentration of 2 µg per ml (Fig. 5). In this case, essentially 100% of the toxin antigen was precipitated by immune sera diluted 1:10, but less than 30% of the antigen was precipitated by normal rabbit sera diluted 1:10.

Serological studies with highly labeled enterotoxin employed at a final concentration of 1 µg per ml resulted in even more sensitive detection of anti-enterotoxin antibody. Again, as Figure 6 indicates, essentially 100% of the toxin was precipitated by various immune sera diluted 1:10, although only 10 to 20% of the antigen was precipitated by 1:10 normal sera.

Figure 7 combines the coprecipitation data obtained with the immune rabbit serum at the three previously mentioned enterotoxin concentrations. Serum titer was taken as the reciprocal of the serum dilution causing precipitation of at least 50% of the labeled toxin antigen. As in the anthrax system, comparisons indicate that the antigen-combining titers of immune sera are inversely related to the antigen concentration employed.

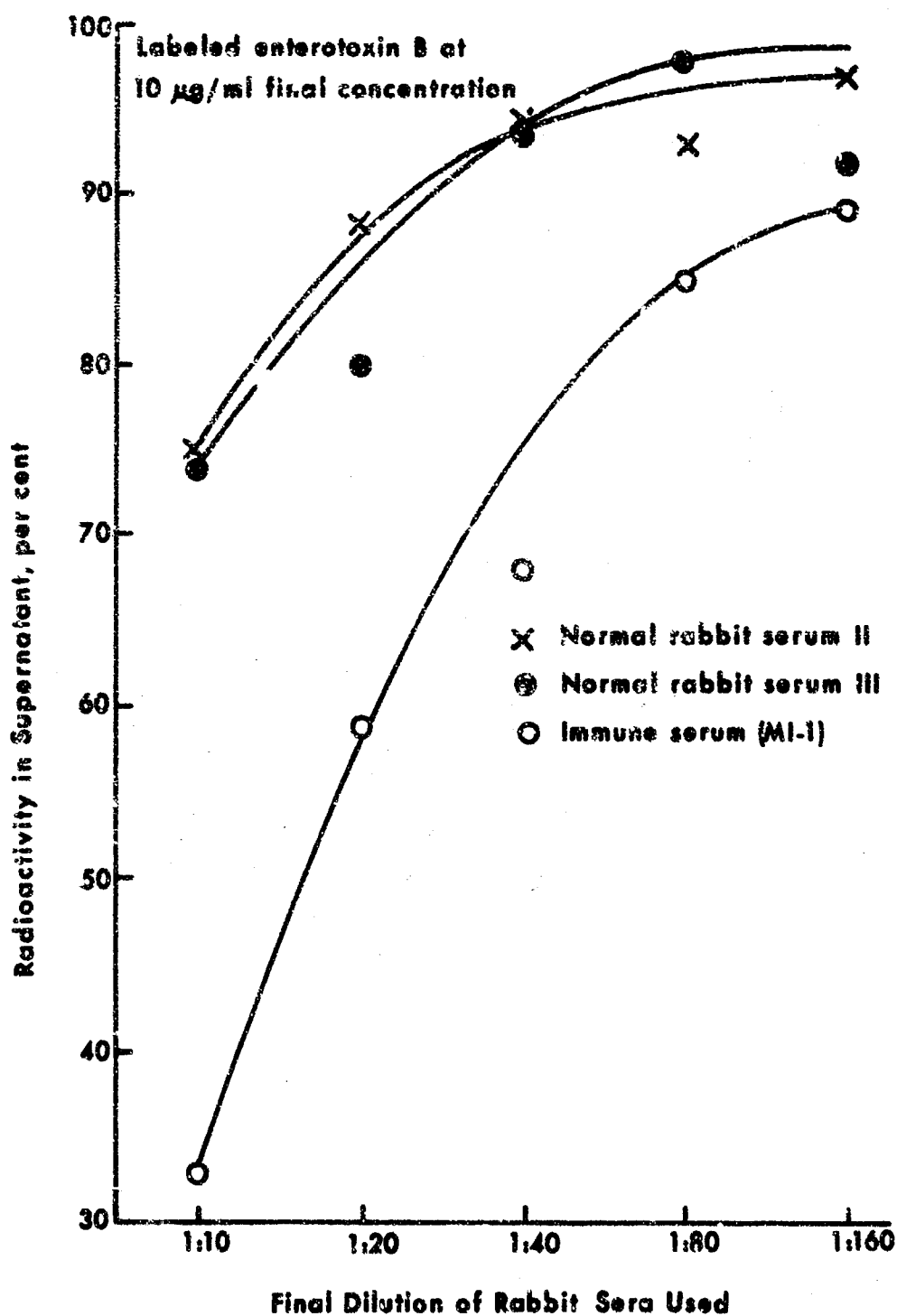


Figure 4. Coprecipitation of Labeled Enterotoxin B and Rabbit Sera Using 3.2 M Ammonium Sulfate.

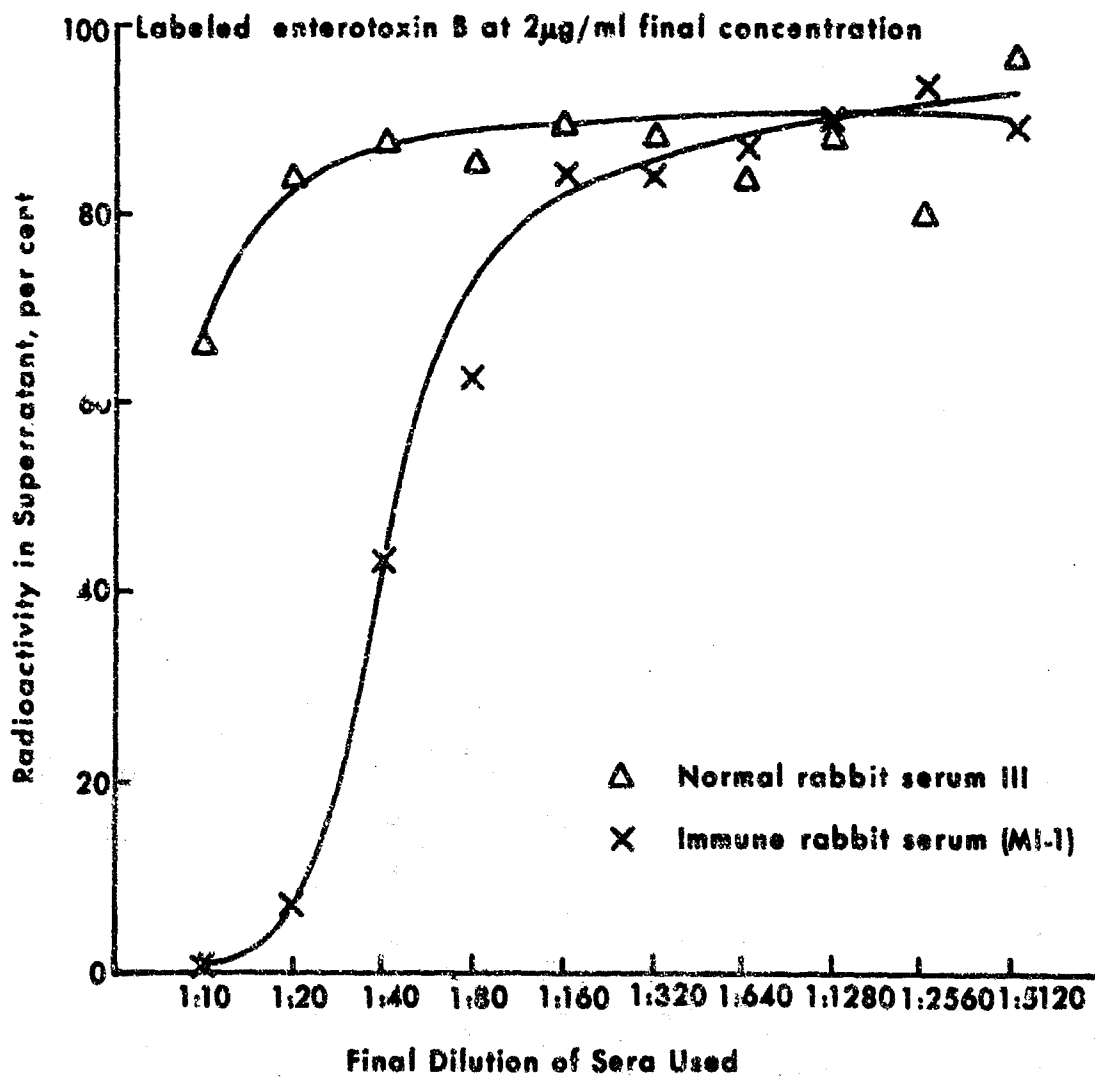


Figure 5. Coprecipitation of Labeled Enterotoxin B and Rabbit Sera Using 3.2 M Ammonium Sulfate.

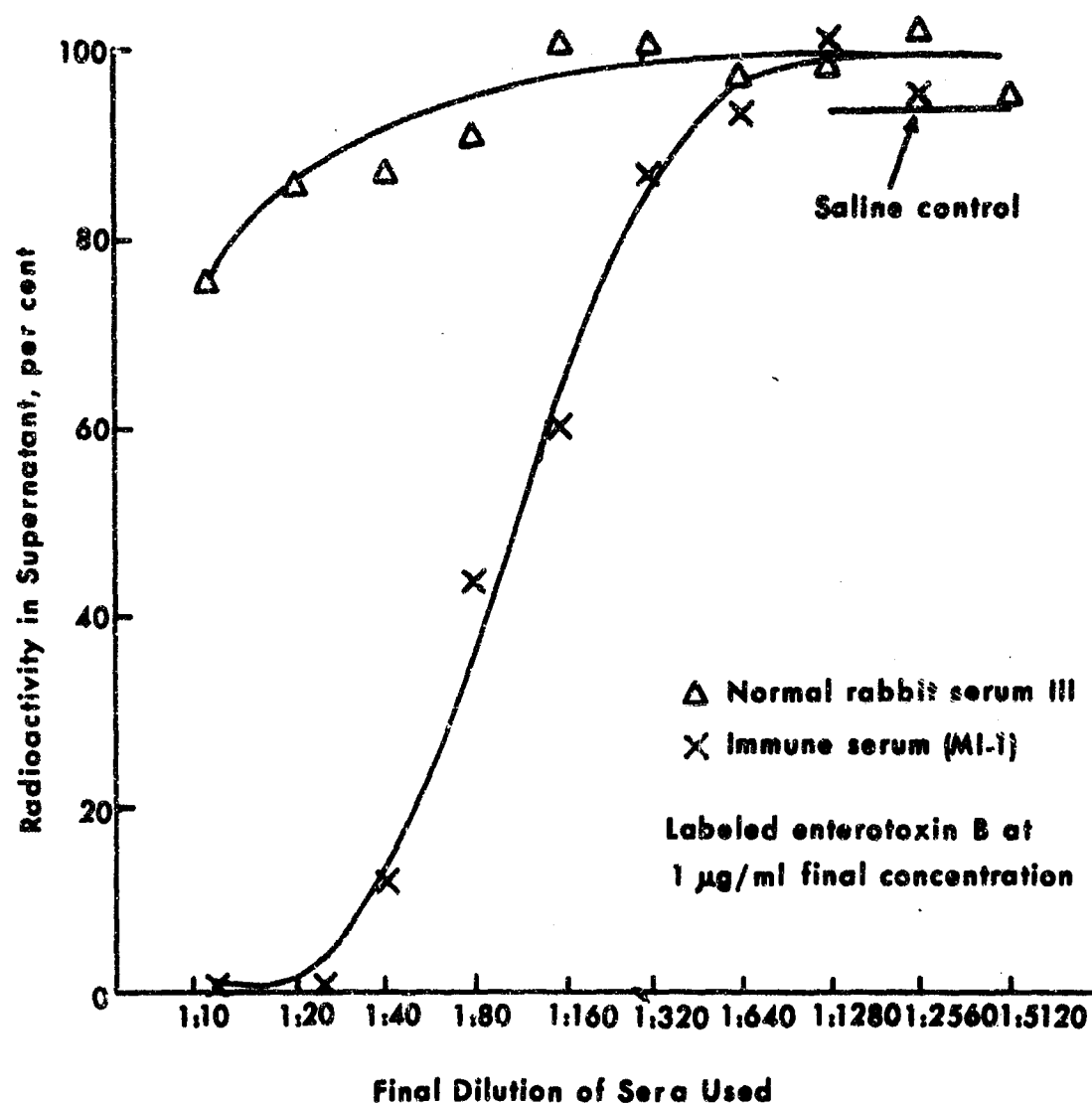


Figure 6. Coprecipitation of Labeled Enterotoxin B and Rabbit Sera Using 3.2 M Ammonium Sulfate.

In summary, the results of this study indicate that the very sensitive and quantitative coprecipitation technique is applicable to the study of antibodies produced in response to microbial antigens. A sensitive method is thus available to test for efficacy of vaccines and immunization.

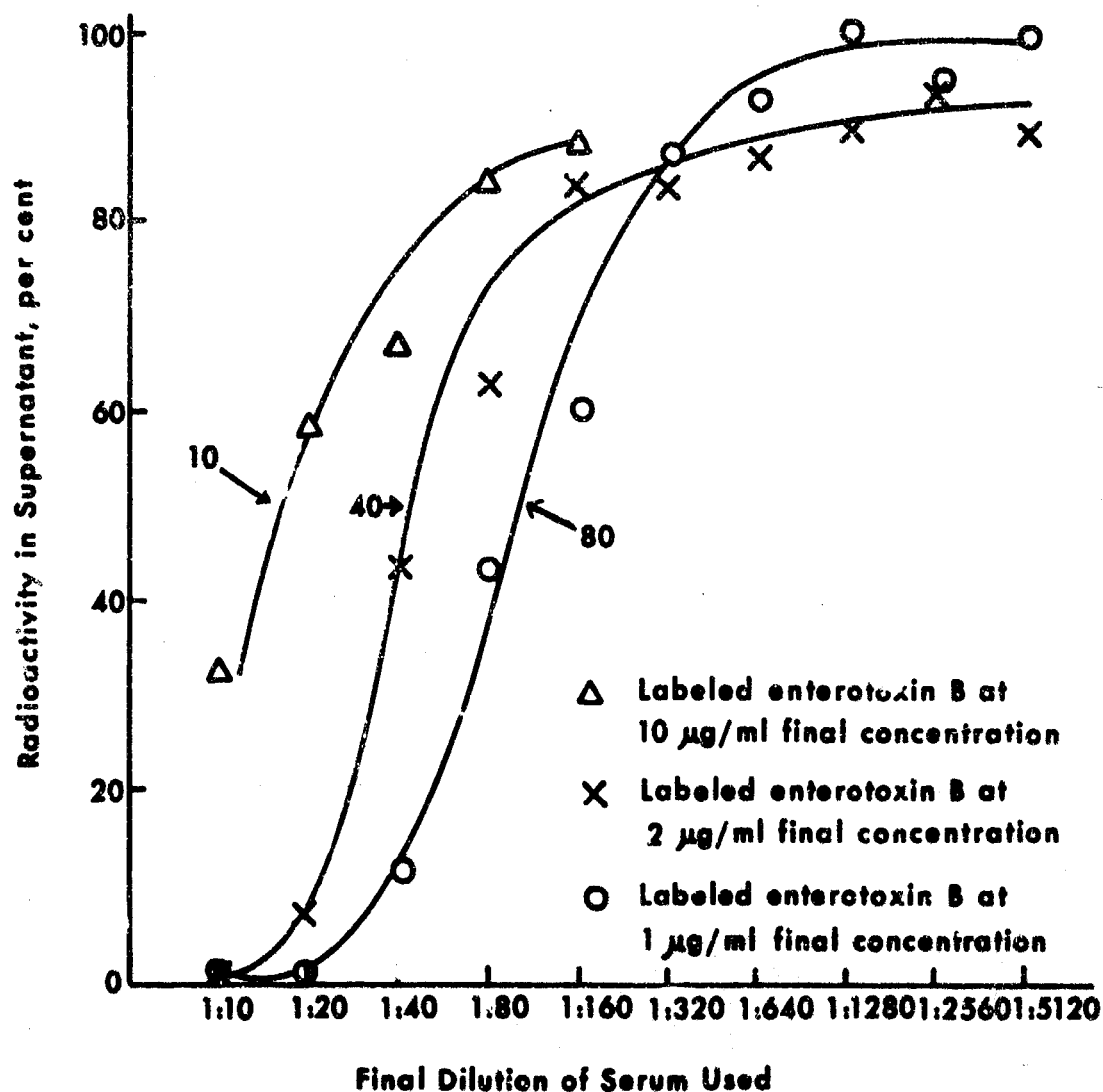


Figure 7. Effect of Labeled Enterotoxin B Concentration on Coprecipitation with Immune Serum.

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