Reports

Immunochemical Studies with Tomato Leaf Proteins

Crude protein preparations from plant tissues frequently give nonspecific precipitation reactions with rabbit serum, which complicate their study by immunochemical methods. Such nonspecific reactions have been avoided by purification of the crude protein preparations or by absorption techniques. Both these procedures remove some of the plant proteins which one may want to study.

In an investigation of the effect of *Fusarium* infection on leaf proteins of tomato, a nonspecific reaction with normal rabbit serum was observed. We wanted to study as many of the plant proteins as possible, hence purification or absorption of the crude protein extracts was not desirable. It seemed that this nonspecific interaction involved serum proteins other than the immune globulins; therefore the fractionation of serum was examined as a possible alternative method of eliminating the nonspecific precipitation reactions. The antigen preparation was obtained from leaves of the tomato variety Bonny Best. The leaves were frozen in liquid air, ground to a fine powder with carbon dioxide, and freeze-dried. Two and one-half grams of the dry powder were extracted at 4°C for 24 hours in a glass tube revolving on a dialysis wheel with 430 ml of 0.05*M* phosphate buffer (*p*H 7.4) containing Merthiolate (1:5000). The cold protein extract was filtered through glass wool and centrifuged in the "Spinco" ultracentrifuge (at 2500 rev/min for 1 hour, with a No. 30 rotor). The crude extract, which contained 121 μg of protein nitrogen per milliliter, was concentrated to approximately 50 ml by pervaporation by means of dialysis (*1*). This concentrated protein extract, containing 588 μg of protein N per milliliter, was brown in color due to the formation of melanin. The melanin was not removed by dialysis; it was prevented from forming in later experiments by addition of ascorbic acid to the extraction medium. The concentrated protein solution [in 0.05*M* phosphate buffer, containing Merthiolate (1:5000)] was stored in the Deepfreeze.

For the immunization of the rabbits, 32 ml of the concentrated protein solution was mixed with 16 ml of a 1-percent aluminum ammonium sulfate solution as an adjuvant [in 0.9-percent saline, containing Merthiolate (1:5000)]. This mixture was adjusted to *p*H 7.0 with sodium hydroxide and diluted to 120 ml to give an antigen solution with 160 μg of protein N per milliliter (approximately 0.1 percent protein).

Before the immunization, a sample of normal serum was obtained from each rabbit. For the immunization, 0.5 ml of antigen solution was injected into each rabbit on four days of the first week, 1.0 ml of antigen solution on each of four days of the second week, 2 ml of antigen solution on four days of the third week, and 3 ml of antigen solution on each of four days of the fourth day. The blood was collected seven days after the last injection, and the serum was stored in the Deepfreeze (*2*).

Part of the sera was subjected to ammonium sulfate fractionation (*2, 3*); another aliquot was used for the preparation of the γ-globulin fractions by alcohol precipitation (*4*). Ring tests were conducted with the antigen and the rabbit serum preparations after various degrees of purification. The antigen solution gave positive ring tests with the homologous antiserum and with normal rabbit serum. This indicated that the whole unfractionated normal serum or antiserum gave a nonspecific precipitation with the crude plant protein antigen. This nonspecific precipitation reaction also occurred with both γ-globulin fractions prepared by ammonium sulfate precipitation. When the antigen was tested with the γ-globulin fractions obtained by alcohol fractionation (*4*), no precipitation reaction could be detected with the γ-globulin preparation from normal serum. There was a strong positive ring test between the antigen and the γ-globulin fraction from the homologous antiserum. The ring tests were repeated with six independently isolated preparations of γ-globulin fractions. Each time the same results were obtained, indicating that the nonspecific precipitation reaction can be avoided if the rabbit serum is sufficiently purified and that this can be achieved by alcohol fractionation of the sera.

To investigate further the specific precipitation reaction between the antigen and the γ-globulins obtained by alcohol fractionation, experiments were carried out to determine the equivalence point and the amount of nitrogen in the precipitate formed by the interaction of the γ-globulin fraction with increasingly large amounts of antigen. Increasingly large amounts of the antigen in 0.5 ml of 0.05*M* phosphate buffer (*p*H 7.4) were added to 0.5-milliliter aliquots of the γ-globulin preparation. After storage for 48 hours under refrigeration, followed by centrifugation (in an International centrifuge, for 30 minutes at 3000 rev/min, with rotor No. 845, refrigerated), the supernatants were decanted and used for the determination of the equivalence point. The precipitates were washed with 5 ml of cold saline, centrifuged as above, decanted, and drained. The washed precipitates were used for the nitrogen determinations (*5*). The point of equivalence for 0.5 ml of γ-globulin preparation was reached when between 80 and 160 μg of antigen had been added. The results of the nitrogen determinations on the precipitates are given in Fig. 1.

The curve in Fig. 1 shows a region of antibody excess with little precipitate formation and an equivalence zone of maximum precipitation, followed by a region of antigen excess in which little or no precipitate is formed. Maximum precipitation, as judged by the amount of precipitated nitrogen, was observed at a level of approximately 160 μg of antigen per sample. The highest amount of antibody N precipitated occurred at a level of 80 μg of antigen per sample; this indicated that the antibody titer of the γ-globulin fraction used was approximately 11 μg of antibody N per milliliter.

The results of this investigation show that tomato leaf proteins give nonspecific precipitates with rabbit serum. Specific precipitation reactions between tomato leaf proteins and the homologous rabbit antiserum can be obtained if the γ-globulin fraction of the antiserum is prepared by alcohol fractionation before being used for the serological tests. The results demonstrate that such a precipitating system shows characteristics commonly found in antibody-antigen reactions, such as specificity, position of the equivalence point relative to the maximum of precipitation, and solubility of the antibody-antigen complex in excess antigen; this

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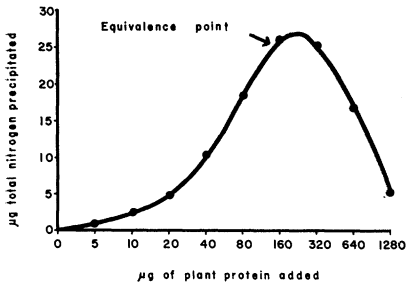

Fig. 1. Amount of nitrogen precipitated in a precipitating system consisting of an alcohol-fractionated-globulin preparation from rabbit antisera and increasingly large quantities of the crude plant protein antigen.

indicates that nonspecific precipitation reactions are absent.

This elimination of the nonspecific precipitation reaction by leaf proteins should make it possible to investigate more adequately by immunochemical methods the proteins from higher plants; such studies, as they relate to pathological conditions, are being continued (*6*).

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References and Notes

1. T. Webb *et al., Can. J. Biochem. and Physiol.* 35, 63 (1957).
2. Grateful acknowledgment is extended to Dr. Dorothy Buchanan-Davidson for advice and assistance in the immunochemical techniques and to Donald E. Slagel for making the ammonium sulfate fractionations.
3. F. E. Kendall, *J. Clin. Invest.* 16, 921 (1937).
4. J. Goldstein and J. W. Anderson, *J. Biol. Chem.* 224, 775 (1957).
5. W. W. Umbreit, R. H. Burris, J. F. Stauffer, *Manometric Techniques and Tissue Metabolism* (Burgess, Minneapolis, 1949), p. 161.
6. This report is published with the approval of the director of the Wisconsin Agricultural Experiment Station. The study was supported by grants from the Herman Frasch Foundation and the Research Committee of the Graduate School, University of Wisconsin.
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Renal Lesions Produced by Group A, Type 12, Streptococci

Group A, types 12, 36, and 3, and group C streptococci (*1*) were grown in Todd-Hewitt broth for 24 hours; each resulting broth culture was inoculated through a glass side arm into a diffusion chamber, and the end of the side arm was sealed in a flame (Fig. 1). The chamber is a modification of the one described by Eschenbrenner and Francis (*2*).

The chambers were fabricated from sheet Plexiglas 3 mm thick. The rectangular pieces with rounded corners measured 20 by 28 mm, and the center hole had a diameter of 12 mm. A hole 1 mm in diameter was drilled in one end, and a 10-mm length of hot capillary tubing was inserted. Membranes of dense porosity (*3*) were glued to each side of the center hole with chloroform-Plexiglas cement. This cement was also used to seal the junction between the glass tubing and the chamber.

Uninoculated and streptococci-containing chambers were placed intraperitoneally in 8-month-old CFW female mice.

It was noted that uninoculated chambers and those containing sterile Todd-Hewitt broth remained in mice for 3 months without evoking renal lesions.

Chambers inoculated with type 12 (nephritogenic) streptococci were implanted intraperitoneally in mice and removed after 24, 48, and 72 hours, respectively. Seven days later the kidneys were removed and the contents of the chamber was cultured to check for contamination. No organisms other than streptococci were found. These kidneys showed proximal tubule necrosis and desquamation of the lining epithelium, moderate thickening of basement membranes, and adhesions between the glomerular tuft and the capsule, with debris and red blood cells in the capsular space. Minimal proliferation of cells of the glomerular tufts was noted (Figs. 2 and 3).

Type 12 (non-nephritogenic) streptococci were implanted as described above, and the chambers and kidneys were removed after the same intervals as in the previous experiments. No evidence of renal lesions was present. To further confirm this, mice were allowed to remain alive for 30 days. At the end of the period no abnormalities were noted in the kidneys.

Type 3 and group C streptococci were used in the above manner without producing renal lesions within 30 days. Isolation of the contents of the chamber revealed pure cultures in each case.

Type 36 streptococci were inoculated into chambers, but leak was purposely made to determine the effect of this type on the mice. Death occurred in 3 to 5 days, with generalized bacteremia; organisms identified as streptococci were isolated from the peritoneal cavity, the blood, and the kidneys. The kidneys were characterized by microabscesses and the picture of acute pyelonephritis. It is interesting to note that implantation of properly sealed chambers bearing type 36 streptococci produced no renal lesions within 1 month.

A streptococcal extract (*4*), prepared after the method of Pappenheimer, when placed in chambers according to the previously described procedure, produced no lesions in the kidneys.

In summary, renal lesions were found in mice bearing diffusion chambers containing nephritogenic type 12 streptococci but not in mice bearing diffusion chambers containing non-nephritogenic type 12 streptococci.

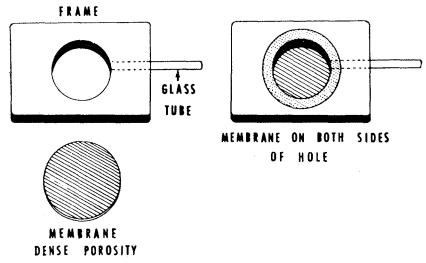

Fig. 1. Modification of chamber described by A. B. Eschenbrenner and R. D. Francis.

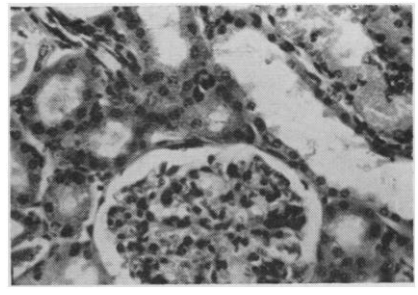

Fig. 2. Photomicrograph of mouse kidney immediately after exposure to type 12 streptococci, in chamber, for 5 days (hematoxylin and eosin stain). (About × 400)

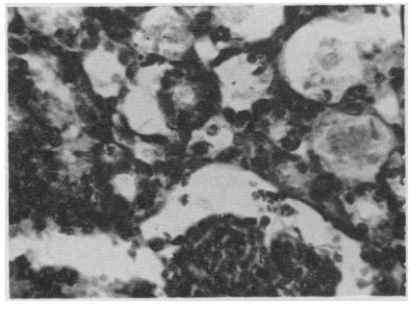

Fig. 3. Photomicrograph of mouse kidney exposed to type 12 streptococci, in chamber, for 48 hours. Kidneys removed 7 days later (hematoxylin and eosin stain). (About × 400)

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References and Notes

1. The following cultures used in this study were furnished by Elaine L. Updyke, Streptococcal Laboratory, Communicable Disease Center, Atlanta, Ga.: Type 12 (nephritogenic) strain DSB-893 isolated from an outbreak of nephritis; type 12 (non-nephritogenic) strain GS-208-4, not known to be associated with nephritis; type 3 strain GS-210-4; type 36 strain SS-269; and type C strain GS-229-4.
2. A. B. Eschenbrenner and R. D. Francis, *Federation Proc.* 15, 514 (1956).
3. The membranes were procured from Schleicher and Schuell Co., Keene, N.H.
4. This extract was prepared from type 12 streptococci, strain DSB-893, by Joseph Schubert, Communicable Disease Center, Atlanta, Ga.

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