PURIFICATION AND CONCENTRATION OF ANTIGENS FOR COMPLEMENT FIXATION BY METHODS OF DIALYSIS, ADSORPTION, AND EXTRACTION.*†

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The complement fixation test of Bordet and Gengou with bacterial antigens on the serum of highly immunized animals has been of value in the identification of bacterial species, but it has never been a practical aid in the diagnosis or prognosis of bacterial infection in man. Although the antigens which have been prepared from syphilitic tissue and even from certain normal tissues have proved to be of great value in the diagnosis of syphilis, none of the antigens which have been prepared from pure cultures of the spirochete isolated from syphilitic tissue has been reliable. On the contrary, they apparently lack reactive capacities that are characteristic for this disease. Thus the nature of the complement fixation reaction of Wassermann is obscure. The so called anticomplementary or nonspecific properties of antigens which have been prepared from pure cultures of the incitants of the bacterial infection have made it difficult or impossible to standardize the test so that the characteristic reactions with the blood serum from cases of infection could be detected or studied to determine their diagnostic or prognostic value.

Prior to his work on syphilis Wassermann studied the complement fixation reaction in tuberculosis, but failed to prepare satisfactory antigens from tubercle bacilli. Recently a number of investigators have reported diagnostic reactions in so many cases of tuberculosis that there can be no question that it is possible to prepare antigens

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from the tubercle bacillus, and in certain stages of the disease the blood serum gives positive reactions with them; but it has not been possible to standardize the test so that it is reliable in the diagnosis of the disease or in the study of the development of immune reactions in different stages of the disease.

Work was begun in 1917, with the view of so standardizing the preparation of the antigens that it would be possible, first, to study the development of immune reactions in experimental tuberculosis in animals and, later, to determine the diagnostic value of the test in human cases of the disease. New methods of dialysis and adsorption were used in the preparation of the antigens by which the antigenic substances were greatly purified and concentrated. The purpose of this preliminary paper is to record the new methods.

Preparation of Standard Immune Serums.

All the cultures were made in a beef infusion broth containing 1 per cent of Fairchild's peptone, 0.5 per cent salt, 0.5 per cent glycerol, and titrated to react 0.5 per cent acid to phenolphthalein. The medium was sterilized fractionally in the Arnold sterilizer. Mass cultures were grown in quart or pint Blake bottles at a temperature of 37.5°C. With some cultures the growth was taken for study as soon as the pellicle had become fully developed, and with others as soon as growth had ceased. After filtration the residue of tubercle bacilli was washed with saline solution and dried partially by suction on the filter. It was then desiccated over concentrated sulfuric acid. This material was then extracted with different substances, such as carbon disulfide, ether, chloroform, and carbon tetrachloride. The fat-free residues from these extractions and also the untreated bacilli were extracted with distilled water, salt solution, sodium hydroxide, hydrochloric acid, and glycerol. The various extracts, together with the filtrates from the cultures, were tested as antigens. This technique was applied to three different strains of tubercle bacilli, a human virulent strain, a human avirulent strain, and a strain of bovine origin. In the production of immune serums by animal inoculation tuberculous organs became available which were extracted by various means and tested for antigenic properties.

The preparation of standard immune serums with these three cultures was a prolonged investigation of experimental infection and immunization of a large number of animals.[1] It involved not only the testing of the inoculated animals at frequent intervals but also a study of the precipitation of the globulin fraction of serum in an attempt to concentrate the antibodies. Finally, sufficiently active immune serums were obtained from horses with all three strains of the tubercle bacillus to give complete fixation of complement with the antigens which were arbitrarily selected to start the work. A single bleeding of any of the horses gave a sufficiently large quantity of serum for the titration of the comparative value of all the antigens.

Antigenic Properties of Different Fractions of the Culture and Methods of Purification and Concentration.

The results of the experiments in which the different fractions of the culture were tested with the standard immune serums must also be briefly summarized. The preparation of antigens by extraction of tuberculous tissues was unsatisfactory. The culture filtrates were so anticomplementary that they could rarely be used. Of all the extracts of the tubercle bacillus which have as yet been tested in our experiments, those with glycerol and, especially, distilled water produced the most active antigens. These active antigens were selected for further study by methods of dialysis and adsorption.

Animal charcoal and globulin (horse serum) were used to adsorb the substances possessing antigenic action. When animal charcoal was added the preparations lost their antigenic activity, but it is impossible to say whether this activity was adsorbed on the charcoal or destroyed as it has been as yet impossible to recover from the animal charcoal any of the antigenic properties. When globulin was added and precipitated with carbon dioxide and the precipitate extracted with alcohol, the substances possessing antigenic action with tuberculous immune serum were adsorbed and recovered in purified and concentrated condition. The so called anticomplementary action which all the original antigens possessed could not be

[1] This work was successfully carried on by Miss B. Johnston and will be recorded more fully in another paper.

detected in the lowest dilutions of the alcoholic extract (1:10) which could be used. The lytic action which some of the original antigens possessed also disappeared.

The technique that was used may be briefly summarized as follows: The original antigen was dialyzed, and horse serum, one part to twenty parts of antigen, was added and the mixture allowed to stand in the incubator for ½ hour. The globulin was then precipitated by passing purified carbon dioxide gas free from hydrochloric acid through the mixture for ½ hour at 37°C. The globulin precipitate was collected, and by shaking it with alcohol the antigenic substances were extracted. The alcoholic extract was concentrated in a vacuum. All the titrations were made in quantities of one-tenth of the original Wassermann test.

In order to eliminate all the dialyzable substances which prevent the adsorption of the antigenic material by globulin, the culture filtrates were dialyzed for at least 5 days. Tests of the antigenic properties at different stages of the process revealed the fact that after dialysis the filtrate became active as an antigen, and lost its anticomplementary action. After precipitation with carbon dioxide and extraction of the globulin precipitate much more active antigens were obtained. The most active antigens, however, were those obtained by this method from the distilled water extracts of the tubercle bacillus.

The following illustrates the degree of concentration and purification. Broth filtrates which were so anticomplementary that they gave no specific fixation, after dialysis were not anticomplementary and 0.02 cc. gave one unit of antigen; that is, complete fixation with the standard quantity of immune serum—two units as determined by a previous titration with one of the crude antigens which were used to standardize the immune serums. After adsorption and concentration 0.0003 cc. gave a unit of antigen. The aqueous extract antigens were anticomplementary in doses of approximately 0.05 to 0.01 cc. The results of purification and concentration of these antigens are even more striking. Before dialysis 0.003 cc. of one of them contained one unit of antigen; after dialysis it was no longer anticomplementary but owing to the increase in volume during dialysis 0.005 cc. contained one unit of antigen. After concentration 0.00015 cc. contained one unit. Expressed in terms of antigenic units per cc. this is an increase from 333 to 6,666.

The results of preliminary experiments with antigens which are used in the diagnosis of syphilis indicate that their antigenic properties may also similarly be purified and concentrated. These new methods thus open up possibilities for more precise study of many phases of infection and immunity than has hitherto been possible.

SUMMARY.

Antigens were prepared from the culture filtrates of tubercle bacilli and by extraction of washed and dried organisms with organic and aqueous solvents and from tissues of organs showing tuberculous lesions.

A comparison of these preparations by means of the complement fixation test showed that the aqueous extracts were most active antigenically.

The antigenic activity of the aqueous preparations and also of the slightly active culture filtrates was increased by means of dialysis and adsorption with serum globulin followed by extraction with alcohol and concentration of the alcoholic extract *in vacuo*.