IMMUNOLOGIC ADSORBENTS. I. ISOLATION OF ANTIBODY BY MEANS OF A CELLULOSE-PROTEIN ANTIGEN*

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For the past several years we have been interested in devising methods that would afford practical procedures for the isolation and purification of antibodies from immune serums and in particular the isolation of non-precipitating antibodies from animal serums and allergic antibodies (reagins) from human serums.[1] The general approach has been to attempt to produce an insoluble protein antigen which would combine specifically with antibody to give a complex that could be dissociated into soluble antibody and insoluble antigen, which could then be separated by centrifugation. Attempts were made to confer insolubility on protein antigens such as ovalbumin and crystalline bovine serum albumin without destroying their antigenicity. Products were obtained by use of the usual denaturing agents, tannic acid, and bifunctional coupling reagents (e.g., tetrazotized benzidine), and also by coupling to an insoluble substrate such as phenolic resins or insoluble proteins (such as fibrin). However, in every case the resulting product either had lost too much native antigenicity (i.e., adsorbed serum always contained antibody which reacted with native antigen), was too soluble, or had too much power of adsorption for non-specific protein.

A most promising antigen has now been prepared by coupling protein to a powdered cellulose (Solka-floc). The general method of synthesizing the cellulose-protein compound consists in conversion of the cellulose to a *p*-nitrobenzyl derivative, reduction of the nitro group, and subsequent diazotization and coupling to protein in the usual manner.

p-Nitrobenzylcellulose.—Powdered cellulose (Solka-floc) was thoroughly washed with dilute acid, dilute alkali, and water, and then dried. Four grams of this material was then mixed with 12 g. of *p*-nitrobenzylchloride and 30 ml. of 40% sodium hydroxide and stirred vigorously at 95°C. (The reacting mixture was cooled during the first part of the exothermic reaction.) After 4 hrs. the mixture was poured into a large excess of cold water and filtered, and the residue was washed with water, with ethanol, and finally with acetone in a Soxhlet extractor. The product contained about 1.5 benzyl groups per glucose unit, and the yield, based on the cellulose used, was close to 100%.

p-Aminobenzylcellulose.—Five grams of the nitro compound was suspended in 50 ml. of ethanol and heated to near boiling. The mixture was then stirred vigorously and 5 g. of sodium hydrosulfite ($Na_2S_2O_4 \cdot 2H_2O$) dis-

solved in water was slowly added. After continued heating of the mixture for about 30 minutes the light yellow product was filtered off and washed with cold water.

Diazotization.—Five grams of the *p*-aminobenzylcellulose was suspended in 10 ml. of 2 *N* HCl, then mixed with 20 ml. of water and chilled in an ice bath. With constant stirring, 0.5 *M* $NaNO_2$ solution was slowly added until a test with KI-starch paper remained positive for 15 minutes after addition of the last portion of nitrite. Stirring was then continued for another 15 minutes and the material was filtered and washed with weakly acid ice water.

Coupling to Protein.—The precipitate of diazonium salt was added to ice-cooled 2% solution of protein in borate buffer pH 8.75. The protein used was crystalline bovine albumin (Armour). The amount of dry protein used for the coupling reaction was generally one-fourth to one-fifth of the dry weight of *p*-aminobenzylcellulose. The coupling mixture was stirred at low temperature for 2 hrs., the pH was adjusted to 7.3, and the mixture was stored at 4°C. for at least 36 hrs. The product was then filtered and washed with large amounts of buffer. The amount of coupled albumin in the preparation used in the present study was about 1.5% of the total weight of the adsorbent.

Covering Unreached Diazonium Groups.—Diazobenzylcellulose seems to be a very stable compound; even after 50 hrs. unreacted diazonium groups are detectable in the coupling mixture. In order to prevent their interaction with serum proteins during adsorption experiments the preparation was allowed to react with β-naphthol, forming a dark red azo dye; proteinazobenzylcellulose was stirred for 30 minutes in 10 volumes of a saturated ice-cooled solution of β-naphthol in borate buffer of pH 8.75, followed by renewed washing on filter with buffer and water.

Since the color of the protein-containing product was only faintly brownish, the reddish tint of the naphthol dye was a qualitative criterion for the completeness of the reaction with the protein. It was assumed that steric hindrance made it impossible for the albumin molecules to couple with all available diazo groups, these being, on the other hand, easily reached by the much smaller naphthol molecule.

The cellulose-albumin complex was found to be an efficient specific adsorbent. Non-specific (non antibody) serum proteins could easily be washed from the adsorbent with 1.0% saline solution. We also found that the chromatographic column had many advantages over the suspension technique, especially for removing all antibodies, including non-precipitating or weakly combining types.

A disconcerting reaction with some preparations of insoluble antigens was the lack of complete removal of all antibody from a sample of serum. This might have resulted from the involvement of certain native haptenic

groups in the coupling reaction or their destruction in some other manner. The cellulose-antigen complexes made with careful control of pH and other coupling conditions, do remove all antibody. Drying the product caused some loss of specific activity.

The antiserum was a pool obtained from rabbits after three to six months immunization with bovine serum albumin (from the batch used for the tests). It contained about 3.39 mg. of precipitating antibody per ml., as determined by the method of Lanni and Campbell.[2]

A 5.0-ml. sample of this serum (containing 17.0 mg. of precipitating antibody) was diluted with 5.0 ml. of 1.0% saline and allowed to pass slowly through a column of about 1.0 g. (wet weight, 0.46 g. dry weight) of the cellulose albumin adsorbent. The packed column was approximately 20 × 8 mm. in size. About 2 hrs. at room temperature was required for the 10 ml. of diluted serum to pass through by gravity only. At the end of this time a small sample of filtrate was removed for antibody determination and the remaining solution was passed through a second similar column. The filtrate from the second column contained no detectable antibody. Analysis of the first filtrate gave a value of about 7.20 mg. total precipitating antibody not adsorbed. This amounts to about 40% not absorbed by the gram of wet adsorbent. If no native antigenic groups had been destroyed in preparation of the adsorbent one would expect that the remaining 40% of antibody could be removed by passage through another column of similar dimensions, which was in fact accomplished.

The acid dissociation method reported by Campbell and Lanni[3] was used to elute the antibody from the adsorbent. The adsorbent was removed from the two chromatographic tubes, suspended in 1.0% saline, and washed and centrifuged three or four times, until the washings were biuret-negative. The pH was then adjusted to approximately 3.2 with 0.1 *N* HCl and the suspension was stirred gently for 60 to 90 minutes at room temperature. The insoluble residue was then resuspended in 1.0% saline and the pH adjusted to 8.2 with borate buffer and tested for precipitating antibody.

Earlier experiments in which denatured albumin was used indicated that elution of antibody which had reacted with remaining antigenic groups was fairly simple. It has been found that ease of elution decreases with increasing length of time that the antibody is allowed to remain on the column. It is important therefore to remove the antibody as soon as possible. When columns are stored in the refrigerator for more than 2 days little or no antibody can be eluted. The following typical elution experiment was carried out with the materials described above. Two samples of 5.0 ml. each of antiserum (3.39 mg. precipitable antibody per ml.) diluted with 5.0 ml. of 1.0% saline were passed through separate columns each containing about 2.0 g. (wet weight) of adsorbent. One column was washed and the antibody was eluted immediately, and the other column

was stored at 4°C. for 12 hrs. before the antibody was eluted. No precipitating antibody could be detected in either filtrate and hence it was assumed that all 17.0 mg. of antibody had been adsorbed in each case. A total of 14.64 mg. of antibody was recovered from the column eluted at once but only 9.50 mg. from the column stored for 12 hrs. at 4°C.

Purity (precipitable antibody/total protein) of recovered soluble material obtained by specific adsorption and elution is high. In the above experiment both columns were eluted at pH 3.2 at room temperature, and the eluate was adjusted to a final volume of 25 ml. The eluate from the first column contained 0.67 mg. of protein per ml., of which 0.59 mg./ml. was precipitable with antigen. The second column gave 0.42 mg. of protein per ml., with 0.38 mg. of precipitable antibody The apparent purity was therefore slightly less than 90% in both these instances, and similar results were obtained with other preparations. It is likely that the eluted protein contains a still larger fraction of antibody, since the antigen-antibody ratio necessary for complete precipitation of antibody may not have been achieved in the precipitation tests.

The question regarding the efficiency of the regenerated (acid-treated) immune adsorbent has not been thoroughly studied, but preliminary tests have indicated that the adsorbing power after one antibody elution is not greatly different from that of the original material. One would expect, however, that some loss would be induced by denaturation of the albumin as well as by lack of complete removal of antibody.

Summary.—Methods are described for the preparation and use of an insoluble antigenic adsorbent obtained by coupling protein to diazotized *p*-aminobenzylcellulose. The data which are presented indicate the practical usefulness of such a method for the isolation and purification of antibody and suggest that such chromatographic techniques might be applied to the separation of antibodies with different reaction capacities.

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