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Use of Radioactive Antibodies for Characterizing Antigens and Application to the Study of Flagella Synthesis

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A simple rapid immunochemical procedure has been developed which provides information about the qualitative and quantitative nature of antigens. It involves the use of purified radioactive (^{125}I-labeled) antibodies. The amount of antibody bound to the antigen is determined by filtering the mixture through diethylaminoethyl (DEAE)-cellulose paper. All of the antigen, as well as the antibody complexed with it, is trapped on the paper, whereas free antibody is removed by repeated washing. This technique has been applied to the study of three immune systems, bovine serum albumin, *Escherichia coli* tryptophan synthetase B protein, and *Bacillus subtilis* flagella. The results obtained by the DEAE-antibody binding technique were comparable, in terms of sensitivity, specificity, and accuracy, to data obtained by microcomplement fixation and precipitin methods. The assay was used to measure the kinetics of flagella regeneration in *B. subtilis*.

One of the major difficulties encountered in studying the synthesis of structural proteins is that they usually lack a specific activity that can be easily measured. In the case of bacterial flagella, the kinetics of synthesis and the nature of the flagellar protein have been determined by direct microscopic observation and by the use of specific antibodies (2, 6; D. Weinstein, H. Koffler, and M. Moskowitz, Bacteriol. Proc., p. 63, 1960). These procedures, however, suffer from having limited application. Some are not strictly quantitative, and others are either tedious or subject to interference by extraneous material in cell extracts.

In another paper, we describe a relatively simple technique for measuring synthesis of flagella by use of purified ^{125}I-labeled antiflagella antibodies (K. Dimmitt, S. Bradford, and M. Simon, *in press*). A mixture of the antibody and bacteria was washed on a membrane filter and the amount of antibody that remained bound to the cells was used as a measure of flagellar protein. Although this technique has allowed precise measurement of the kinetics of flagella regeneration on the bacteria, it cannot be used to estimate purified flagella or the flagellar subunit protein.

In the present study, we have extended this approach by taking advantage of the differential adsorbtion of the antigen and antibody onto ion-exchange resin. The amount of antibody bound to the antigen is determined by filtering the mixture through diethylaminoethyl (DEAE) cellulose paper. All of the antigen, as well as the antibody complexed with it, is trapped on the paper, whereas free antibody is removed by washing the filter paper. This is a generally useful immunochemical test that provides information about the qualitative and quantitative nature of the antigen. Experiments designed to characterize the assay by use of three antigens, bovine serum albumin (BSA), *Bacillus subtilis* 168 flagella, and tryptophan synthetase B protein, will be described.

MATERIALS AND METHODS

Antibodies and antigens. B. subtilis 168 flagella were purified and antisera were prepared as previously described (K. Dimmitt et al., *in press*). The anti-BSA was an ammonium sulfate precipitate derived from pooled rabbit antiserum and was donated by S. J. Singer of this department. BSA was purchased from Pentex Corp., and other serum albumins were a gift from D. Benjamin, Scripps Clinic and Research Foundation. The tryptophan synthetase B protein and the antibody were provided by S. Mills of this department.

The purified antibodies were prepared according to the procedures of Freedman et al. (1). Immune precipitates were formed by incubating the antigen and antibody at 37 C for 1 hr (antigen concentra-

tions were at or near equivalence). The complex was stored at 4 C overnight and then washed three to five times with 0.01 M phosphate buffer (*p*H 6.8) containing 0.15 M sodium chloride. The complex was dissolved overnight in deionized 8 M urea at 4 C. The separation of antigen and antibody was then effected by chromatography on DEAE-cellulose in 8 M urea, 0.01 M phosphate (*p*H 7.4). Antibody concentrations, as determined from absorbance measurements (optical density at 280 mμ), were: anti-BSA, 2.4 mg/ml; anti-tryptophan synthetase B protein, 1.4 mg/ml; and anti-*B. subtilis* 168 flagella, 2.5 mg/ml. After extensive dialysis against 0.01 M phosphate buffer, 0.15 M sodium chloride, the purified antibody was stored at −20 C.

The reaction mixture (2 ml) for iodination contained approximately 1 mg of protein per ml in 0.1 M phosphate buffer. The antiflagella antibody was treated with 10 mc of carrier-free ^{125}I, whereas only 1 mc was used with the other antibodies. The reaction was carried out according to the methods described by McConahey and Dixon (4). After the iodination was terminated, rabbit γ-globulin was added to a final concentration of 0.5 mg/ml, and the total reaction mixture was applied to a column of Sephadex G-25 (1 × 25 cm) and eluted with 0.01 M phosphate buffer (*p*H 6.8), 0.05 M sodium chloride. The protein was completely separated from unbound iodine.

Preparations of iodinated antibody were stored at −20 C and used for 2 to 3 months. The results to be presented have not been corrected for the decay of ^{125}I.

DEAE assay procedure. The assay was carried out by incubating 0.10 ml of iodinated antibody and 0.15 ml of appropriately diluted antigen for 30 min at room temperature. Duplicate 0.1-ml samples were added to a double thickness of DEAE cellulose paper (Whatman DE81), presoaked and washed in buffer containing 0.025 M tris(hydroxymethyl)aminomethane (Tris), *p*H 7.2, 0.05 M NaCl, and 100 μg of rabbit γ-globulin per ml. In the assay for flagella, the concentrations of Tris and NaCl were doubled to 0.05 and 0.1 M, respectively. Samples were allowed to adsorb onto the DEAE paper for 5 min and then washed four times each with 4 ml of the buffer. In all of the filtration steps, precautions were taken to prevent air from passing through the filter, since this results in increased background counts. Samples were filtered on a 10-port vacuum filter apparatus.

Complement fixation was performed in accordance with the procedures described by Levine and Wasserman (7). The membrane filter assay for regeneration of flagella is described elsewhere (K. Dimmitt et al., *in press*).

Flagella regeneration. B. subtilis 168 M try⁻ or 168 M try⁻ lys⁻ was grown in minimal medium (5) supplemented with 0.10% Casamino Acids and 30 μg/ml of the appropriate growth factors. The cells were sheared in a VirTis Omnimixer (The VirTis Co., Inc., Gardiner, N.Y.) at 13,000 rev/min for 30 sec to remove flagella and they were then collected by centrifugation and resuspended in minimal medium. At various times after the initial shearing, samples were removed from the culture and sheared again. The cells were discarded and the supernatant fluid was diluted appropriately and tested for flagella. The concentration of bacteria was determined by plating on Penassay medium and by turbidity measurements.

We have chosen to express our results as percentage regeneration. This is calculated by dividing the amount of radioactive antibody bound to antigen derived from 10^6 bacteria at any time during regeneration by the amount of antibody bound per 10^6 bacteria for the fully flagellated bacteria before initiating regeneration.

RESULTS

Antibody binding and antigen concentration. The relationship between antibody binding and antigen concentration for the three test systems is shown in Fig. 1–3. All three immune systems show the same general characteristics. At low antigen concentration, when sufficient antibody was added, binding was directly proportional to the amount of antigen added. This linear response reflects the binding of a maximal number of antibody molecules to each antigen molecule. Thus, when the antibody concentration was increased, there was no change in the initial slope of the binding curve, but the linear region was extended to higher antigen concentrations. Under these conditions, the linear portion of the binding curve can be used as a calibration curve for the determination of antigen concentrations in crude extracts of cells.

At relatively high concentrations of antigen (Fig. 1–3), most of the available antibody was combined. The maximal amount of antibody bound varied with the different antibody preparations and was usually 60 to 80% of the total

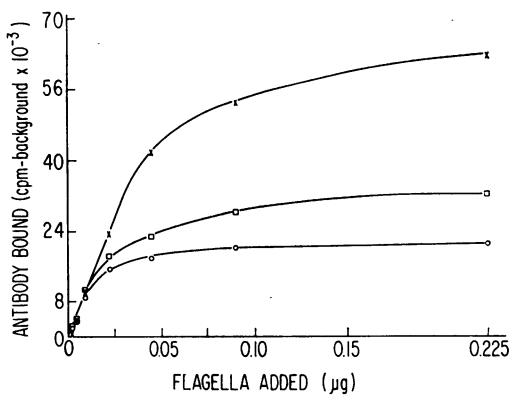

FIG. 1. *Dependence of the amount of iodinated flagella antibody bound upon flagella concentration. Determinations were carried out at* (×) *1:20,* (□) *1:40, and* (○) *1:60 dilutions of* ^{125}I*-antibody; background levels were 10,950, 5,350, and 3,000 counts/min, respectively.*

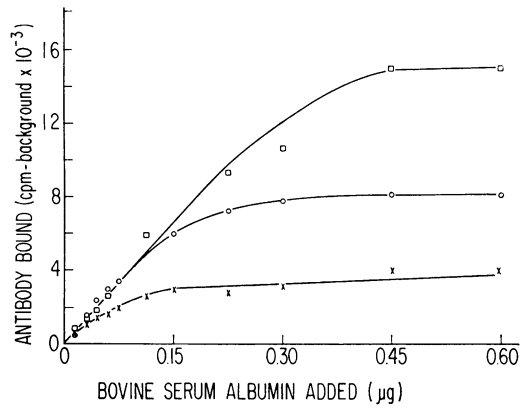

FIG. 2. *Dependence of the amount of iodinated BSA antibody bound upon BSA concentration. Assays were carried out at* (□) *1:10,* (○) *1:20, and* (×) *1:40 dilutions of* ^{125}*I-antibody; background levels were 4,750, 1,900, and 450 counts/min, respectively.*

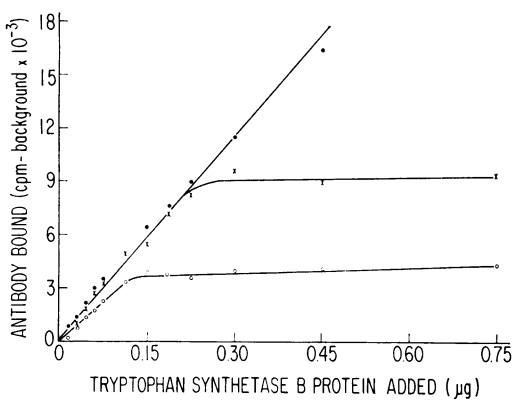

FIG. 3. *Dependence of the amount of iodinated tryptophan synthetase B protein antibody bound upon tryptophan synthetase B protein concentration. Determinations were carried out at* (●) *1:5,* (×) *1:10, and* (○) *1:25 dilutions of* ^{125}*I-antibody; background levels were 3,000, 1,500, and 700 counts/min, respectively.*

radioactivity. In the plateau region of the curve, binding was essentially independent of antigen concentration and proportional to antibody concentration (Fig. 4). Under these conditions, the relative titer of new preparations of antibody could be determined.

Measurement of cross-reacting antigens. The assay can also be used to measure the degree of cross-reaction with related antigens. Figure 5 shows the binding of anti-BSA to bovine, sheep, pig, and human serum albumins. The plateau region of the binding curve is a measure of the maximal amount of antibody that reacts with each antigen. Forty-three per cent of the antibody that reacts with the homologous antigen also reacts with sheep serum albumin, 13% with pig serum albumin, and 6% with human serum albumin. When the antibody concentration was raised threefold, the values were similar: sheep, 39%; pig, 17%; and human, 8%. In studies of cross-reaction measured by precipitin tests, other investigators have found (8) values of 74, 31, and 14% (5.3:2.2:1) for the three cross-reacting antigens. Although the values are different from

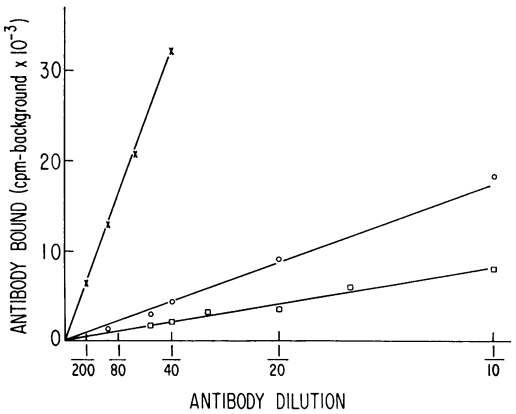

FIG. 4. *Amount of* ^{125}*I-antibody bound as a function of antibody dilution. In all three systems, a constant excess level of antigen was used. Symbols:* ×, *Bacillus subtilis flagella-anti-B. subtilis flagella at 0.3 μg of flagella;* ○, *BSA-anti-BSA at 0.5 μg of BSA;* □, *tryptophan synthetase B protein-anti-tryptophan synthetase B protein at 0.38 μg of tryptophan synthetase B protein.*

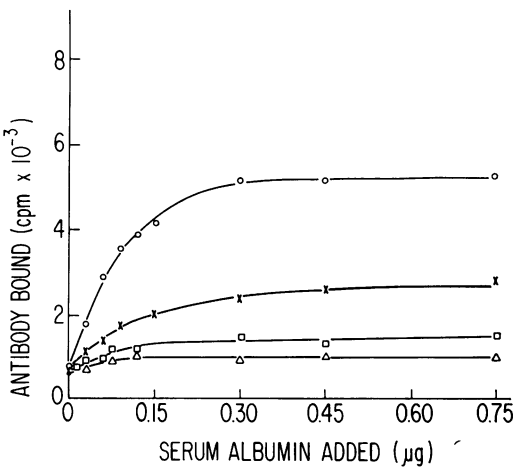

FIG. 5. *Cross-reaction of heterologous serum albumins with BSA* ^{125}*I-antibody. The antibody was used at a 1:35 dilution and the antigens were:* ○, *BSA;* ×, *sheep serum albumin;* □, *pig serum albumin;* △, *human serum albumin.*

those determined in our experiments, the relationship among the antigens is essentially the same (5.8:2.1:1).

The cross-reaction of flagellin, the protein subunit of the flagellar filament, with antiflagella antiserum is shown in Fig. 6. Thirty per cent of the antibody directed against the whole flagellum also reacts with the subunit. In studies of antibodies made to *Proteus vulgaris* flagella (K. S. Read, M. Moskowitz, and H. Koffler, Federation Proc., p. 609, 1956), a similar degree of cross-reaction was found with the flagellin subunits (20%), when measured by precipitin techniques.

We have also (in collaboration with S. Mills) measured cross-reaction of tryptophan synthetase B apo-protein (minus pyridoxal phosphate) with the anti-tryptophan synthetase B protein antibody. The results obtained by the DEAE-antibody binding technique were consistent with measurements made by complement fixation.

Kinetics of flagella regeneration. A comparison between the measurement of flagella regeneration made by the DEAE-antibody binding technique and by complement fixation is shown in Fig. 7 and 8 and in Table 1. The material sheared from the bacteria was treated with acid and measured as flagellin. The absolute amounts of flagella-like material determined by both assays is similar. The major discrepancies occur early during regeneration, when the concentration of antigen is low.

Finally, in other experiments, the degree of regeneration was determined by antibody binding to whole bacteria and by the DEAE method with sheared flagella filaments (Fig. 9). Both techniques gave identical results.

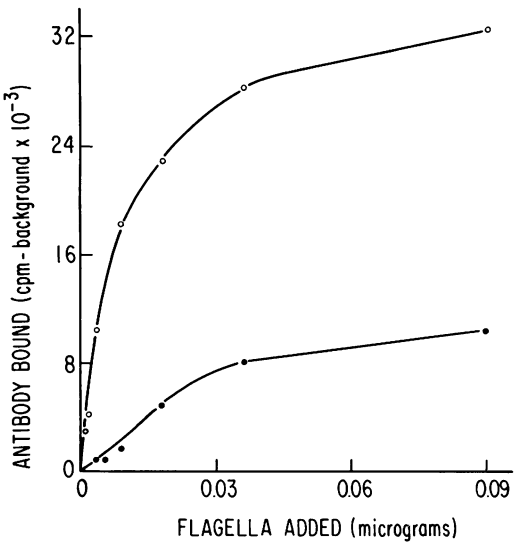

FIG. 6. *Cross reaction of flagellin with anti-flagella antibody. The determination was carried out at 1:40 ^{125}I-antibody dilution. Symbols: ○, flagella; ●, flagellin. Background level was 5,500 counts/min.*

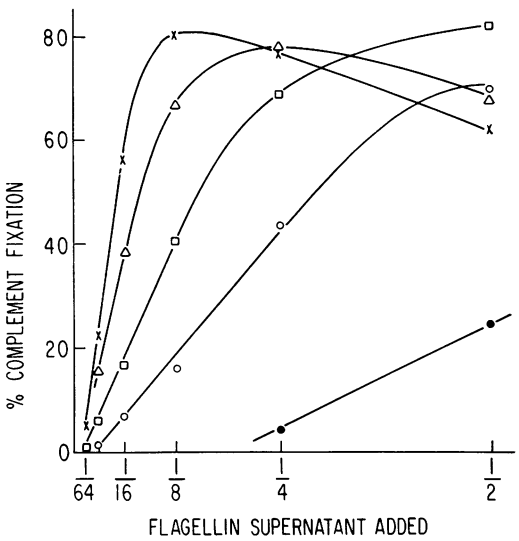

FIG. 7. *Measurement of flagella regeneration by complement fixation. The regeneration experiment was performed as described in Materials and Methods. The flagella-containing supernatant fluids were adjusted to pH 2.4 and incubated for 15 min at room temperature. They were centrifuged at 20,000 × g for 15 min, and the pH was readjusted to 8.0. The reaction of flagellin was measured with a 1:2,000 dilution of serum 2543-1. Symbols: ×, before shearing; ●, after shearing; ○, at 10 min; □, 20 min; △, 30 min.*

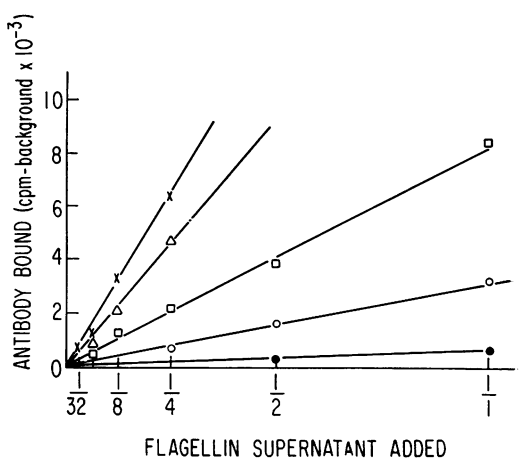

FIG. 8. *Measurement of flagella regeneration by the DEAE-antibody binding assay. Symbols: ×, before shearing; ●, after shearing; ○, 10 min; □, 20 min; △, 30 min.*

TABLE 1. *Kinetics of flagella regeneration*[a]

Time	Complement fixation			DEAE-antibody binding		
	Flagella per ml of culture	Flagella per 10^9 bacteria	Regeneration	Flagella per ml of culture	Flagella per 10^9 bacteria	Regeneration
	μg	μg	%	μg	μg	%
Before shearing	0.24	2.2		0.25	2.3	
After shearing	0.015	0.11	5	0.0085	0.063	2.7
10 min	0.048	0.33	15	0.038	0.26	11
20 min	0.090	0.53	24	0.087	0.51	22
30 min	0.17	0.81	37	0.19	0.90	38

[a] The values in this table were obtained by comparing the data presented in Fig. 7 and 8 with standard calibration curves prepared with purified flagellin (the protein concentration was determined by the Kjeldahl nitrogen method).

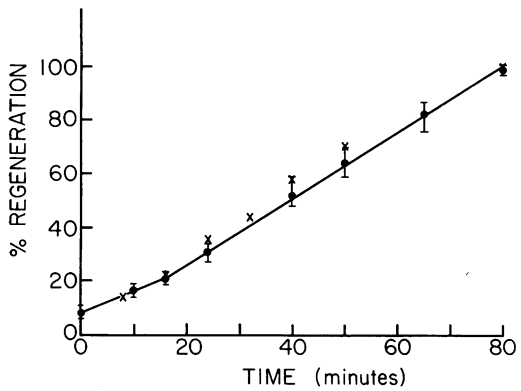

FIG. 9. *Comparison of flagella regeneration, as measured by ^{125}I-antibody binding to bacteria (membrane filtration) and flagella removed from the bacteria (DEAE cellulose assay). Symbols: ●, average of five regeneration experiments measuring antibody binding to bacteria; ×, measurements made by the DEAE-antibody binding method.*

DISCUSSION

The DEAE-antibody binding technique that we have described has the advantages of being easy to use and highly specific. It is particularly applicable to measuring antigens in crude extracts, where anticomplementary activity interferes with complement-fixation assays and the attachment of the antigen to other cellular components interferes with precipitin methods. The amount of extraneous material that can be tolerated appears to be limited only by the capacity of the ion-exchange paper. The level of detection of antigen can be adjusted by varying the degree of labeling of the antibody and by increasing the concentration of extract. The lower limit of detection is established by the amount of background counts that are bound to the paper in the absence of antigen. In the three systems that we have investigated, the results obtained by the DEAE-antibody binding technique were comparable, in terms of sensitivity, specificity, and accuracy, to data obtained by complement-fixation and precipitin methods.

Although the antigens that we have used are adsorbed to DEAE, the use of other adsorbants is not excluded. We have attempted to establish a ribonuclease-antiribonuclease system using carboxymethyl cellulose paper. However, even though the free antigen did adhere to the paper, the antigen-antibody complexes did not. This was probably due to the low molecular weight of the ribonuclease. In antibody excess, a number of antibody molecules are bound and these molecules may mask the antigen and prevent adsorbtion to the resin.

The major limitation of the DEAE method stems from the antibody purification and iodination steps. These procedures result in the inactivation of part of the antibody (from 20 to 40%). The heterogeneity thus introduced does not affect the application of the technique to measurements of antigen concentration. It does, however, make it difficult to determine low levels of cross-reaction and it inhibits a more rigorous interpretation of the antibody binding curves. There are a number of approaches that may be used to minimize these effects; e.g., the iodination can be performed with the antigen-antibody complex (3), different methods can be used to purify the antibody (9), and other labeling methods that are less prone to oxidizing the antibody can be used.

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LITERATURE CITED

1. FREEDMAN, M. H., L. SLOBIN, J. B. ROBBINS, AND M. SELA. 1966. Purification of antibodies in urea. Arch. Biochem. Biophys. **116**:82–90.
2. KERRIDGE, D. 1963. Synthesis of bacterial flagella. J. Gen. Microbiol. **33**:63–76.
3. LANGENBERG, W., AND D. SCHLEGEL. 1967. Autoradiography with ^{125}I-labeled antibodies. Virology **32**:167–170.
4. MCCONAHEY, P. F. AND F. DIXON. 1966. A method of trace iodination of proteins for immunologic studies. Intern. Arch. Allergy Appl. Immunol. **29**:185–189.
5. SPIZIZEN, J. 1958. Transformation of biochemically deficient strains of *Bacillus subtilis* by DNA. Proc. Natl. Acad. Sci. U.S. **44**:1072–1078.
6. STOCKER, B. A. D., AND J. C. CAMPBELL. 1959. Removal of flagella by non-lethal methods. J. Gen. Microbiol. **20**:670–685.
7. WASSERMAN, E., AND L. LEVINE. 1961. Quantitative microcomplement fixation and its use in the study of antigen structure. J. Immunol. **87**:290–296.
8. WEIGLE, W. O. 1961. Heterologous antigen-antibody reactions. J. Immunol. **87**:599–607.
9. WELIKY, N., H. H. WEETALL, R. V. GILDEN, AND D. H. CAMPBELL. 1964. The synthesis and use of some insoluble immunologically specific adsorbants. Immunochemistry **1**:219–229.