Isolation of Anti-dinitrophenyl Group Antibodies with a Triply Substituted Cross-reacting Antigen*

William C. Cheng† and David W. Talmage

From the Department of Microbiology, University of Colorado School of Medicine, Denver, Colorado 80220

(Received for publication, December 4, 1964)

Antibody to the 2,4-dinitrophenyl determinant is one of the most easily obtained and probably the best characterized anti-hapten antibody. This antibody has been used for studies involving fluorescence quenching in the antibody-hapten reaction (1), affinity labeling of the active sites on the antibody (2), the binding capacity of reduced antibody (3), the unfolding and renaturation of univalent antibody fragments (4), and a number of other immunological characterizations (5-8). The anti-dinitrophenyl employed for all of these studies was purified by the procedure of Farah, Kern, and Eisen (9) which recovers one-fourth of the total precipitable antibody in the antiserum. The antibody not recovered by this procedure probably includes antibody with a higher binding affinity for the antigen used to separate the antibody from the other serum proteins. The present paper describes an alternative method which permits the isolation of 70% of the anti-DNP antibodies. The method is based on the complete precipitation of anti-DNP from antiserum with a weakly interacting, triply substituted cross-reacting antigen. This is followed by nearly complete elution of bound antibody by the hapten, dinitrophenol, which can be subsequently removed from the antibody by means of anion exchange chromatography. The anti-DNP obtained from this method is 90% reprecipitable with the homologous DNP-antigen.

Experimental Procedure

Reagents—2,4-Dinitrophenyl sulfonic acid sodium salt, 2,4-dinitrophenol (Eastman), and \( \epsilon N \) 2,4-dinitrophenyl-l-lysin (Sigma) were recrystallized three times from water before use. Analytical grade \( \sigma \)-nitrofluorobenzene, \( \tau \)-nitrofluorobenzene (Sigma), and \( \epsilon N \), \( \epsilon T \) -trinitrobenzene sulfonic acid (Nutritional Biochemicals), nitroanilines (K and K Laboratories), and streptomyein sulfate (Squibb) were used without further purification. DEAE-cellulose was obtained from Mann Research Laboratories. Dowex 1 anion exchange resin, Ag 1 X8, 200 to 400 mesh, was purchased from Bio-Rad Laboratory; Sephadex G-25, coarse grade, was from Pharmacia, and Amberlite IRA-400, Cl\(^{-}\) form, from Mallinckrodt Chemicals. Human \( \gamma \)-globulin, bovine serum albumin, and bovine \( \gamma \)-globulin were purchased from Pentex, Inc.

Antigens—Maximally substituted 2,4-dinitrophenyl-BSA and nearly maximally substituted DNP-BGG and DNP-HGG were prepared and analyzed according to the method of Eisen, Belman, and Carsten (10, 11). The conjugated proteins contained about 40 to 60 moles of DNP per mole of protein.

Immunization—Rabbits were immunized with DNP-BSA in Freund's complete adjuvant. Each rabbit received 10 mg of protein in 2 ml of emulsion of which 0.5 ml was injected into each of the four footpads. The rabbits were bled from the ear vein 6 weeks later, followed by weekly bleedings. Unless otherwise noted, the antisera used were pooled.

Antiserum—Most of the work was carried out with serum 1 which was pooled from 15 rabbits. Pooled serum 2 from 60 rabbits was used for performing the precipitin tests shown in Fig. 1, and the study of net yield and binding constants of purified antibody. Ten different pooled sera were used for the study of the effectiveness of the triply substituted antigen in the precipitation of anti-DNP.

Cross-reacting Antigen—\( \sigma \)-Nitrophenyl-BGG and \( \tau \)-nitrophenyl-BGG were prepared by dropwise addition of ethanol solutions containing 0.1 m \( \sigma \)-nitrofluorobenzene or \( \tau \)-nitrofluorobenzene, respectively, to 100 ml of a 1% BGG solution containing 1% Na\(_2\)CO\(_3\) until the molar ratio of the reagents to BGG reached 60:1. The reaction mixtures were stirred continuously at 37\(^\circ\) for 24 hours in the dark. 2,4,6-Trinitrophenyl-BGG was prepared by the addition of 1 g of 2,4,6-trinitrobenzenesulfonic acid to 100 ml of a 1% BGG solution containing 1 g of Na\(_2\)CO\(_3\). The mixture was shielded from light and stirred continuously for 0 hours at 37\(^\circ\). The three conjugated proteins were purified by first passing through a Sephadex G-25 column (2.9 \times 50 cm) followed by the passage through an Amberlite (IRA-400, Cl\(^{-}\) form) column (2.9 \times 10 cm). Both resins had been preconditioned with phosphate buffer. The approximate number of substituents on each of the conjugated proteins was determined by measuring the protein concentrations and the nitrophenyl groups in solutions of the conjugated proteins in 0.1 n NaOH. The former was determined by the micro-Kjeldahl analysis and the latter was computed from the ultraviolet absorption of the conjugated proteins at 350 m\(_\mu\) (\( \sigma \)-nitrophenyl), 390 m\(_\mu\) (\( \tau \)-nitrophenyl), and 340 m\(_\mu\) (2,4,6-trinitrophenyl) by assuming that the absorbance at these wave lengths was entirely due to the substituted groups. The standard solutions for these measurements were made of \( \sigma \)-nitroaniline, \( \tau \)-nitroaniline, and 2,4,6-trinitroaniline in 0.1 n NaOH. The number of substituents

* Supported by Research Grant AI-04152 from the National Institute of Allergy and Infectious Diseases, National Institutes of Health, United States Public Health Service. A preliminary report was presented at the 146th National Meeting of the American Chemical Society, January 19 to 24, 1964.

† Candidate for the Doctor of Philosophy degree, 1965. This paper is a portion of a thesis being submitted for the above degree.

The abbreviations used are: anti-DNP, antibody directed against the dinitrophenyl group; DNP-, dinitrophenyl-; BGG, human \( \gamma \)-globulin; T-HGG, its triply substituted form; BSA, bovine serum albumin; BGG, bovine \( \gamma \)-globulin; T-BGG, its triply substituted form.
calculated from this method was on the average 50 moles of substituent per mole of BGG in all three cases.

The preparation of triply substituted BGG and triply substituted HGG was performed in the following manner. An ethanol solution containing 0.1 m o-nitrofluorobenzene and 0.1 m p-nitrofluorobenzene was added dropwise to a 1% BGG or HGG solution containing 1% NaCO₃ until the molar ratio of each of the reagents to protein was 15:1. The reaction mixture was stirred continuously for 24 hours at room temperature. At this point the protein solution should have a yellow color. 2,4,6-Trinitrobenzene sulfonic acid, 1 g, was then added to the yellow protein solution. The mixture was continuously stirred for 6 hours at 37°. The dark orange, conjugated proteins were purified by passage through the columns of Sephadex and Amberlite as described for the preparation of the three singly substituted antigens. The protein contained about 10 o-nitrophényl and p-nitrophényl and 30 to 40 2,4,6-trinitrophenyl groups per molecule. The "T-proteins" obtained in this way bind strongly to DEAE cellulose at neutral pH; they are also readily precipitated with streptomycin sulfate at a concentration of 40 mg per ml.

Unless otherwise mentioned, phosphate buffer, 0.01 m, pH 7.4, in 0.15 m NaCl was used throughout all operations.

DEAE-cellulose chromatography was performed according to the method of Sober and Peterson (12). Ultracentrifugation was performed with a Spinco model E ultracentrifuge at 59,780 rpm and 24°.

Iodination—Anti-BSA and complement were removed from 5 ml of antiserum to DNP-BSA by adding a slight excess of BSA, incubating at 35° for 30 min, leaving at 4° overnight and centrifuging at 1400 X g. The supernatant was precipitated with 40% saturated (NH₄)₂SO₄. The precipitate containing anti-DNP and other rabbit γ-globulins was resuspended in buffer, dialyzed, and then iodinated with ¹²⁵I at a molar ratio of iodine to globulin of less than 1 by the method of Talmage, Baker, and Akeson (13). Before the protein was added to the reaction mixture, nitrous acid was removed by addition of urea and the pH of the mixture was brought to approximately 6.5. The iodinated globulin was dialyzed to remove free iodide. It was then added to approximately 100 ml of the original antiserum and anti-BSA was again removed. The antiserum prepared in this way was referred to as ¹²⁵I anti-DNP serum.

¹²⁵I Rabbit γ-Globulin Standards—A small portion of the iodinated antiserum was precipitated with 40% saturated (NH₄)₂SO₄. The ¹²⁵I globulin precipitated was dialyzed and then fractionated on a DEAE-cellulose column. The first protein peak obtained at column volume was collected and the protein concentration was determined by absorption at 280 mp, taking the extinction coefficient at 280 mp as 1.46 per mg per ml at pH 7.4 (1). Standards containing 1 ml of varying concentrations of the ¹²⁵I γ-globulin were made and the radioactivity in each sample was determined in a well type scintillation counter.

Precipitin Reaction with DNP-BGG or DNP-HGG—Equal volumes of varying concentrations of DNP-BGG or DNP-HGG were added to equal volumes of ¹²⁵I antiserum. Controls were made by adding different concentrations of NaCO₃-treated BGG or HGG. The mixtures were incubated at 37° for 1 hour followed by 24 hours standing at 2°, and were then centrifuged. The specific precipitates obtained were washed with 2 ml of ice cold phosphate-NaCl solution buffer three times. The precipitates were then dried and dissolved in 0.25 m acetic acid.

The radioactivity of each of the precipitates was determined and followed by a measurement of ultraviolet absorption at 280 mp and 353 mp. In order to obtain a measure of antibody in the precipitate, correction for the absorbance contributed by the antigen was made according to the method of Farah, Kern, and Eisen (9). Measurement of antibody was also made by comparison of the radioactivity in the precipitate with the ¹²⁵I rabbit γ-globulin standards.

Precipitin Reaction with Cross-reacting Antigens—The precipitin tests were performed in the same manner as described above except that in most cases only the ¹²⁵I rabbit γ-globulin standards were used for determining the amount of antibody precipitated. In the case of precipitin tests with the triply substituted antigens, the amount of antibody precipitated was also measured by ultraviolet absorption of specific precipitates in 0.25 m acetic acid at 280 mp and 340 mp. The calculation used was by the method of Farah, Kern, and Eisen (9). In some cases, the total nitrogen precipitated by the antigen was analyzed by the micro-Kjeldahl nitrogen determination.

The supernatants obtained in the zone of maximal precipitation with T-BGG or T-HGG were tested with DNP-BGG, DNP-HGG, and DNP-JSA.

Dissolution of Specific Precipitates—Precipitates were prepared according to the method just described. To each tube containing about 1 mg of anti DNP, 2 ml of varying concentrations of the sodium salt of 2,4-dinitrophenol, pH 7.4, was added. The mixture was stirred continuously with a micro stirring bar. After 1 or 2 hours, streptomycin sulfate was added to a concentration of 40 mg per ml. The mixture was stirred for another 15 min and then centrifuged at 1400 X g for 1 hour at 10°. Each precipitate after decantation was washed with 2 ml of streptomycin-phosphate buffer solution. The wash was then added to the original supernatant. The precipitate was dissolved in 4 ml of 0.1 N NaOH. The radioactivity of the precipitate and supernatant tubes were then determined.

Fluorescence Quenching—Fluorimetric titrations for measuring the binding of hapten to antibodies were done with an Amino-Bozman spectrophotofluorometer modified to incorporate a thermostatically controlled cell housing. Antibody (40 μg) in 1 ml was titrated with a total of 0.18 to 0.2 ml of a 6 X 10⁻⁶ m hapten solution added in increments of 0.01 to 0.02 ml. The entire procedure followed the method of Eisen and Siskind (8) and Eisen (11). The average intrinsic association constant, Kₐ, was calculated from the Sips equation of Karush (14)

$$\log \frac{r}{n - r} = a \log e + a \log K_a$$

where r is moles of hapten bound per mole of antibody at free hapten concentration, e, and n is the valence of the antibody (i.e., 2); a is a constant (heterogeneity index). For each titration, log r/n − r was plotted against log e; Kₐ can be obtained from the intercept, and a was obtained from the slope.

RESULTS

Comparison of the quantity of anti-DNP precipitated was made by the determination of radioactivity and ultraviolet ab-

*We wish to express gratitude to Dr. Charles W. Parker of Washington University School of Medicine, St. Louis, Missouri, for helpful discussions concerning the technique of fluorescence quenching.
Comparison of antibody precipitated by determination of radioactivity and ultraviolet absorption on same precipitates

<table>
<thead>
<tr>
<th>Antigen added</th>
<th>Antibody precipitated determined by</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Absorbance</td>
</tr>
<tr>
<td>mg</td>
<td>mg</td>
</tr>
<tr>
<td>0.14</td>
<td>0.21</td>
</tr>
<tr>
<td>0.10</td>
<td>0.32</td>
</tr>
<tr>
<td>0.05</td>
<td>0.34</td>
</tr>
<tr>
<td>0.03</td>
<td>0.26</td>
</tr>
</tbody>
</table>

The results of dissociation of $^{125}$I anti-DNP from DNP-HGG and T-HGG are summarized in Fig. 3. Ninety per cent of the antibody precipitated with T-HGG was readily eluted by the hapten, 2,4-dinitrophenol, while only 40 to 50% was eluted from DNP-HGG precipitates. Attempts were made to recover the remaining antibody bound to T-HGG by elution with 0.1 N HCl. The amount of protein obtained from this treatment accounted for only 3% of the total protein that was initially precipitated from the original antiserum.

**Purification Procedure**—The usual preparation consisted of triply substituted antigens were always the same in tests with 10 different pooled antisera.

The precipitating capacity of the cross-reacting antigens was compared to DNP-HGG. $p$-Nitrophenyl-BGG, 2,4,6-trinitrophenyl-BGG, and $o$-nitrophenyl-BGG precipitated from 20 to 60% of the radioactivity precipitable with the homologous antigen, DNP-BGG. However, when the three cross-reacting haptenes were substituted on the same BGG or HGG, the resulting antigen (T-BGG or T-HGG) precipitated 100% of the radioactivity precipitable with DNP-HGG or DNP-BGG. The results are shown in Figs. 1 and 2. The supernatants obtained at the point of maximum precipitation with T-BGG or T-HGG gave negative precipitin tests with DNP-BGG, DNP-HGG, and DNP-BSA. Control tests on antiserum with 1% Na$_2$CO$_3$-treated BGG and HGG were also negative. The total maximum amount of antibody precipitated with the DNP-substituted and 200 ml of antiserum from which anti-BSA and complement had been removed by prior precipitation with BSA. The antiserum was mixed with the amount of T-HGG or T-HGG required to give maximal precipitation. The mixture was incubated at 37° for 1 hour with gentle stirring, followed by standing at 2° for 24 hours. The precipitate collected after centrifugation at 1400 × g for 1 hour was washed with 25 ml of ice-chilled phosphate buffer in 0.15 M NaCl. At least two washings were performed after the last wash giving a negative test for protein with 10% trichloroacetic acid. The washed precipitate was then resuspended in 100 ml of phosphate buffer containing 0.1 M 2,4-dinitrophenol. The suspension was incubated at 37° for 1 to 2 hours with continuous gentle stirring. After this period, streptomycin sulfate was added.

We routinely used T-BGG rather than T-HGG. The amount of antibody precipitated was determined by measuring the specific precipitates at 250 and 340 nm.
Fig. 2. Precipitin reactions of anti-DNP with DNP-HGG and T-HGG

Fig. 3. Dissolution of anti-DNP from DNP-HGG and T-HGG precipitates
**Fig. 4.** Ultracentrifugal analysis of purified anti-DNP. The concentration of antibody was 0.3%. Centrifugation was carried out at 59,780 rpm at 24°. Schlieren patterns were photographed at 8-min intervals at a bar angle of 60°. The first picture was taken 1 min after reaching full speed.

Immunizing antigen

\[
\text{O}_2N - \text{HGG (T-HGG)}
\]

Antiserum (anti-DNP-BSA) + BSA

\[
\text{BSA}
\]

![Antibody precipitate](image)

Supernatant (anti-DNP) + BSA

\[
\text{BSA}
\]

![Precipitate](image)

Washed precipitate

\[
\text{BSA}
\]

![Wash](image)

1. Elution with 0.1 M 2,4-dinitrophenol, pH 7.4, 37°
2. Streptomycin sulfate (40 mg per ml)

![Discard](image)

Effluent

![Residual precipitate](image)

TABLE II

<table>
<thead>
<tr>
<th>Specific precipitation of purified antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNP-HGG added</td>
</tr>
<tr>
<td>mg nitrogen</td>
</tr>
<tr>
<td>None</td>
</tr>
<tr>
<td>0.01</td>
</tr>
<tr>
<td>0.36</td>
</tr>
<tr>
<td>0.29</td>
</tr>
<tr>
<td>0.24</td>
</tr>
<tr>
<td>0.21</td>
</tr>
<tr>
<td>0.18</td>
</tr>
</tbody>
</table>

was added to a concentration of 40 mg per ml. The mixture was stirred continuously for 15 min and was subsequently centrifuged at 1400 × g for 1 hour at 10° to yield a supernatant solution containing at least 90% of the anti-DNP initially precipitated. Free 2,4-dinitrophenol and streptomycin were removed by means of a Sephadex G-25 column (2.5 × 50 cm) coarse grade. The lightly colored bound anti-DNP recovered was passed through a column (2.5 cm in diameter), the top 5 cm of which contained DEAE-cellulose and the lower 30 cm Dowex 1 (Cl⁻ form). The DEAE-cellulose was used to remove trace amounts of T-HGG which might still persist in the eluted antibody solution since T-HGG strongly adsorbs to the cellulose resin under the described conditions. The column chromatography was performed at room temperature and the flow rate of the Dowex 1 column was adjusted to 0.3 ml per min. The effluent antibody solution obtained should be colorless and it was usually further concentrated by means of 50% saturated (NH₄)₂SO₄ precipitation, followed by dialysis in phosphate buffer. At this point, the net yield was 70% of the antibody originally precipitated. In preliminary work, purified antibody solution was tested with DEAE-cellulose without the prior treatment of the resin and was also subjected to ultracentrifugation (Fig. 4). Both methods demonstrated that the purified anti-DNP was exclusively 7S γ-globulin. If labeled antiserum was used, the radioactivity per mg of the final purified antibody agreed well within 5% with the ³H rabbit γ-globulin standards. An outline of the purification scheme is shown in Fig. 5.

Purification of Antibody—About 90% of the ³H purified anti-DNP was precipitable with DNP-HGG on the basis of radioactivity determination on the precipitate, and 88% or occasionally 90% on the basis of spectral determinations of the supernatant at 280 and 360 nm. The results are shown in Table II and Fig. 6. It is noted that the purified antibody alone has a broader zone of maximum precipitation than antiserum. This is probably due to interference by nonantibody proteins, for example, albumin and other low molecular weight substances in the serum. In some cases, the total precipitated by DNP-HGG and T-HGG were analyzed by the micro-Kjeldahl nitrogen determination. The results were in close agreement with both the radioactivity and the ultraviolet absorption data.

Effect of Purification Procedures on Specific Binding of Antibody—Antibody purified by the present method (antibody 2C) and antibody 2E purified by the method of Farah, Kern, and Eisen (9) from the same pooled serum of 60 rabbits were com-

4 This was done by dialysis in 20 liters of phosphate buffer overnight in the cold, followed by centrifugation to remove a very small amount of insoluble material.
Fig. 6. Specific precipitation of purified $^{125}$I anti-DNP

Fig. 7. Fluorometric titrations at 20° of antibody preparation 2C obtained by the present purification procedure and antibody 2E obtained by the method of Farah, Kern, and Eisen (9) from the same pooled antiserum. A, 1 ml samples containing 40 μg of antibody were used for each titration, taking the extinction coefficient at 280 mμ as 1.46 per mg per ml at pH 7.4. The molecular weight of the antibody was taken as 160,000. Fluorescence is corrected for the solvent fluorescence (9%) and for the volume of hapten added. B, the data of A are plotted according to Equation 1. The fluorescence-quenching data are calculated according to the method of Eisen and Siskind (8) and Eisen (11).
The lysine side chain also contributes a small but significant energy of binding. Furthermore, the triply substituted antigen has some unique properties. It is insoluble in streptomycin and also binds strongly to DEAE-cellulose at conditions where antibody bound to the T-BGG or T-HGG could be readily dissociated from the precipitate with a low affinity hapten, 2,4-dinitrophenol. The triply substituted antigen was removed from the soluble hapten-antibody complex by precipitation with streptomycin and a diethylaminoethyl cellulose column. The streptomycin and free hapten were subsequently dissociated from the antibody with an anion exchange column. The procedure yielded 70% of the originally precipitable antibody in the antiserum. Ninety per cent of the anti-DNP precipitated is not eluted by 2,4-dinitrophenol from the T-BGG or T-HGG precipitates according to the radioactivity data. This amount of antibody remaining in the precipitate may be due to either denatured labeled antibody or an incomplete elution of the antibody within an interval of 1 to 2 hours. The latter explanation seems unlikely since even the high affinity hapten, \( \epsilon \text{-N-DNP-lysine} \) gives only 93% elution of the anti-DNP from the precipitate.

The net yield of purified anti-DNP from our purification scheme was 70% of the antibody initially precipitated. The purified antibody from the present isolation procedure seems to contain a fraction of high affinity antibody for the \( \epsilon \text{-N-DNP-lysine} \) determinant. However, a definitive conclusion can only be drawn when a successful attempt has been made to separate the heterogeneous antibodies into discrete populations and analyze each population individually. It is suggested that a similar principle may be used for the isolation of antibody in other hapten-antibody systems.

<table>
<thead>
<tr>
<th>Antibody preparations</th>
<th>( K_a )</th>
<th>2,4-Dinitrophenol</th>
</tr>
</thead>
<tbody>
<tr>
<td>2C</td>
<td>50</td>
<td>0.5</td>
</tr>
<tr>
<td>2E</td>
<td>14.3</td>
<td>2.11</td>
</tr>
</tbody>
</table>

Some substitution at other sites, e.g., imidazole and phenolic groups, cannot be excluded.