THE PREPARATION AND SOME PROPERTIES OF PURIFIED ANTIBODY SPECIFIC FOR THE 2,4-DINITROPHENYL GROUP*, ‡

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In the past 25 years most efforts to prepare highly purified antibodies have been motivated by two aims: (*a*) the identification of antibodies as proteins, in particular as γ-globulins; (*b*) the study of the stoichiometry and energetics of antibody-antigen and antibody-hapten interactions. Additional needs for relatively large quantities of purified antibodies are becoming more and more evident, *e.g.* (*a*) in structural studies of antibodies by enzymatic and chemical means, (*b*) for use as authentic reagents in the identification of trace amounts of proteins in studies on the biosynthesis of antibodies and γ-globulins, (*c*) for use as reagents in studies of allergic responses. It is, in fact, clear that highly purified antibodies are now, and will become increasingly regarded, as indispensable reagents for large numbers of studies on the structure, function, and biosynthesis of antibodies.

In the present account we wish to describe a procedure which has been developed for the procurement of gram quantities of antibodies specific for the 2,4-dinitrophenyl group in satisfactory yield (about 40 per cent) and in a high state of purity. In the following report, the purified antibody has been applied to the study of wheal and erythema responses in human skin (1). In other studies, the principles involved in the purification scheme have been applied to detection and measurement of microsomal antibodies (2), and of antibodies synthesized by isolated cells (3). Some physical properties of the purified anti-

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body are given in a report on the stoichiometry and energetics of its interaction with haptens and antigen (4).

Materials and Methods

Low Molecular Weight Dinitrophenyl Reagents.—2,4-dinitrobenzenesulfonate (Na salt), 2,4-dinitrophenol, and 2,4-dinitrophenylacetic acid were obtained from Eastmen Kodak Co., Rochester, New York, and were recrystallized several times from water; melting points (uncorrected; microblock) for the latter two substances were 113° and 186–187°, respectively. ε-DNP-lysine[1] and ε-DNP-aminocaproic acid were prepared and characterized previously (5). DNP-glycine was made by Porter's method (6) and crystallized from water; its melting point was 200–207°.

A mixture of DNP-peptides was prepared from dinitrophenyl-bovine γ-globulin (see below) which was treated with one-half its weight of pepsin at pH 1–2. After 60 minutes at 37°, perchloric acid was added (0.5 M), and the precipitate discarded. The yellow supernatant was neutralized with KOH in an ice bath, the insoluble salt discarded, and the soluble material was extracted three times with *n*-amyl alcohol to remove ε-DNP-lysine (7), and two times with peroxide-free ether. The residual aqueous material, after concentration at reduced pressure, consisted of a mixture of ε-DNP lysyl peptides. Its absorption maximum and minimum were, respectively, 360 mμ and 300 mμ. For determination of the concentration of ε-DNP lysyl residues, the molecular extinction coefficient of ε-DNP lysine was used (17,400).

1-C^{14}-2,4-dinitrophenol was prepared by heating 1-C^{14}-2,4-dinitrochlorobenzene (134,000 C.P.M. per μmole; Tracer lab, Boston, Mass.) in N NaOH for 2 hours at 100° (8). The solution was then extracted three times with ether (to remove residual dinitrochlorobenzene), acidified to pH ∽1, and extracted again with ether. The latter ether extract was taken to dryness and the residue dissolved in a minimal volume of 0.01 M phosphate–0.15 M NaCl, pH 7.5.

Preparations of Antigens.—Bovine γ-globulin, very nearly maximally substituted on ε-NH_2 groups of lysine residues by 2,4-dinitrophenyl groups, was used both to immunize rabbits and to precipitate antibodies from antisera. These substituted proteins (DNP-BγG) were prepared and analyzed as previously described (9, 7).[2] The several conjugates used had from 54 to 65 moles DNP per 160,000 gm. BγG. When used as a precipitating antigen, DNP-BγG was diluted with 0.14 M NaCl, 0.01 M phosphate, pH 7.4, usually to about 600 μg./ml. At higher concentrations of NaCl, the DNP-BγG preparations were insoluble to variable but appreciable degrees. In the buffered salt solution specified, the preparations were sufficiently soluble to be perfectly satisfactory for precipitin assays, the antigen blank tubes having less than 1 per cent the absorbance of the specific precipitates analyzed (see Table I).

Preparation of Antisera.—Immunizing emulsions of the Freund type (10), were made with one volume of arlacel A, four volumes of mineral oil (bayol F) containing 100 μg. air-dried *Mycobacterium butyricum* per ml., and five volumes of DNP-BγG in 0.15 M NaCl, 10 mg. pro-

[1] Abbreviations: DNP is used generically for the 2,4-dinitrophenyl group combined with amino acids or proteins. ε-DNP lysine, ε-2,4-dinitrophenyl lysine; ε-DNP aminocaproate, ε-2,4-dinitrophenyl aminocaproic acid; BγG, bovine γ-globulin; DNP-BγG, 2,4-dinitrophenylbovine γ-globulin.

[2] The conjugates were ordinarily freed of low molecular weight contaminants by dialysis against running tap water for 5 to 7 days. More rapid purification of heavily substituted DNP-proteins may be accomplished by adjusting the pH to about 4, and by washing the precipitated protein with 0.1 M acetate, pH 4. For small reaction volumes, anion exchange columns (IRA-400, Cl^- form) have proven rapid, convenient, and efficient in removing low molecular weight contaminants from DNP-proteins (7).

TABLE I

*Specific Precipitation of Purified Antibody**

Antigen added§	Absorbance‡				Antibody precipitated‖		Antigen precipitated‖	
	Supernatants		Precipitates					
	278 mμ	360 mμ	278 mμ	360 mμ				
μg.					mg.	per cent	μg.	per cent
None¶	1.591	0.013	0.003	0.001				
85.5	0.383	0.013	0.374	0.148	0.89	76	84	98
143	0.163	0.012	0.480	0.245	1.06	91	142	99
171	0.151	0.016	0.520	0.296	1.07	91	169	99
200	0.142	0.020	0.534	0.338	1.08	92	197	99
256	0.153	0.037	0.584	0.434	1.07	91	251	98
114**	0.422	0.720	0.001	0.001				
200**	0.760	1.312	0.001	0.000				

* The lyophilized preparation of antibody had been kept for 2 months at 4° before use in this experiment. 1.19 mg. of antibody protein was added to each tube. Total volume in each vessel was 1.0 ml. The tubes were incubated for 60 minutes at 37°, then overnight at 4° before centrifugation. Supernatants were read without dilution. Precipitates were washed once with 0.15 M NaCl, air-dried, and dissolved in 4.0 ml. 0.25 M acetic acid.

‡ Corrected only for cuvet blanks. Values are averages for duplicate tubes. Agreement between duplicates was better than ±2 per cent.

§ DNP-BγG; about 60 moles DNP per 160,000 gm. BγG.

‖ Based on supernatant analyses. Indistinguishable results were obtained by analyses of 0.25 M acetic acid solutions of washed precipitates, providing the assumption is made that in these solutions the antibody absorbance at 360 mμ is 5 per cent of its absorbance at 278 mμ (rather than ~1 per cent as used in the corresponding supernatant analyses; see the reading for the antibody control tubes in horizontal line 1 of this table). Although the dissolved precipitates give perfectly clear solutions, this assumption of increased turbidity is probably justified by the observations described in footnote 4.

¶ Antibody control tubes.

** Antigen controls (no antibody added). The "supernatant" absorbances in these tubes provide two of the constants required for calculating the amounts of antigen and of antibody: (*a*) the ratio of absorbances 278 mμ/360 mμ is 0.58; (*b*) $E_{1\text{ cm.}}^{1\text{ per cent}}$ for the antigen at 360 mμ is 65.

tein per ml. Each rabbit was injected at one time with 0.2 ml. of the foregoing emulsion in each footpad.[3] 6 to 8 weeks later the animals were bled. Occasionally, after bleeding, the rabbits were injected intravenously with 0.5 to 1 mg. of DNP-BγG in 1 ml. of 0.15 M NaCl, and

[3] Antisera with somewhat higher anti-DNP antibody concentrations have been obtained quite regularly in subsequent work by injecting footpads with twice the volume of complete adjuvant (a total of 1.6 ml. per rabbit), containing the same quantity of antigen (4 to 5 mg. DNP-BγG). Arlacel A (mannide monooleate) and bayol F were generously supplied by the Atlas Powder Co., Wilmington, and the Penola Oil Co., New York, respectively.

were then bled again on 3 consecutive days, beginning 5 days later. All bleedings were by cardiac puncture and the sera were frozen until used. Sera having similar antibody concentrations (as determined by precipitin analyses with DNP-BγG) were pooled. The pools used in the present study varied from 1.5 to 3 mg. anti-DNP antibody per ml.

Measurement of Precipitating Anti-DNP Antibodies.—Reaction mixtures were set up in duplicate with a constant volume of antiserum and variable amounts of DNP-BγG. After incubation (60 minutes at 37° followed by 1 to 2 days at 4°) precipitates were collected and washed twice at 2° with 0.15 M NaCl. Washed precipitates were air-dried, dissolved in 0.25 M acetic acid, and absorbancies were determined (Beckman spectrophotometer, model DU) at 278 mμ and at 360 mμ, the respective maxima for protein and DNP groups in the precipitates. In previous work, dilute NaOH was used as the solvent for precipitates (11) because extinction values given by proteins above pH $\backsim$10–11 are higher than at lower pH values. However, the absorbancies in alkali change slowly with time (12), and acetic acid, as suggested by Gitlin (13), is preferable since absorbancies are stable in this solvent. After correction for antigen and antibody blanks, the absorbance at 278 mμ was corrected for the antigen contribution. The latter correction was based on the absorbance of DNP-BγG at 360 mμ and at 278 mμ. A secondary correction was made for the contribution of antibody to the 360 mμ absorbance.[4]

The following observations indicate that although the same kind of conjugated protein (DNP-BγG) was used for both immunization and precipitation of antisera, the precipitates obtained were almost entirely specific for the DNP group: (*a*) ϵ-DNP lysine inhibited pre-

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[4] More explicitly, the amounts of antibody and of antigen in a dissolved specific precipitate are calculated as follows:—

$$B = \frac{V}{E_B}\,(a - R[b - f(a - Rb)]),$$

and

$$G = \frac{V}{E_G}\,(b - f[a - Rb]),$$

where B is antibody, G is antigen, V is volume (ml.), E_G is the extinction coefficient of antibody at 278 mμ (absorbance/mg. protein/ml.), E_B is the extinction coefficient of antigen at 360 mμ (absorbance/mg. protein/ml.), a is absorbance at 278 mμ, b is absorbance at 360 mμ, R is ratio of absorbances for antigen at 278 mμ/360 mμ, and f is ratio of absorbances for antibody at 360 mμ/278mμ.

R varies with degree of substitution of DNP-protein; in near maximally substituted DNP-BγG it is usually 0.58 to 0.60 at pH 7 (see Table I). The same value is assumed to apply in 0.25 M acetic acid solutions of specific precipitates.

f is about 0.01 for antibody at neutral pH (Table I). When specific precipitates are dissolved in 0.25 M acetic acid, f is, however, taken to be 0.05. The assumption that f is here increased is justified by the following evidence which indicates that antigen-antibody aggregates exist in these solutions: (*a*) DNP-BγG (heavily substituted) is insoluble in 0.25 M acetic acid, yet specific precipitates containing this antigen dissolve readily in this solvent giving optically clear solutions. (*b*) Purified anti-DNP antibodies bind ϵ-DNP lysine, specifically and strongly, at acid pH values, down to 1.5 (4).

The foregoing method of calculation was used also to measure the amounts of antibody and antigen in supernatants of precipitin reactions made with purified antibody (*e.g.* Table I), and in this case f is 0.01.

cipitation almost completely (> 90 per cent; see Fig. 2). (*b*) ϵ-DNP lysine and ϵ-DNP aminocaproic acid dissolved specific precipitates almost completely (> 90 per cent; see Fig. 2). (*c*) Native BγG, and BγG denatured by prolonged shaking with K_2CO_3 (to simulate the conditions used for preparing DNP-BγG), precipitated only 5 per cent as much antibody from the antisera as did DNP-BγG.

EXPERIMENTAL AND RESULTS

Purification Procedure.—The scheme used for the preparation of purified antibody is outlined in Fig. 1. In a typical small scale preparation, 100 ml. clarified antiserum (18,000 × *g*, 1 hour) was mixed with that quantity of DNP-BγG required to give maximal precipitation. After incubation for 1 hour at 37°, the mixture was kept over-

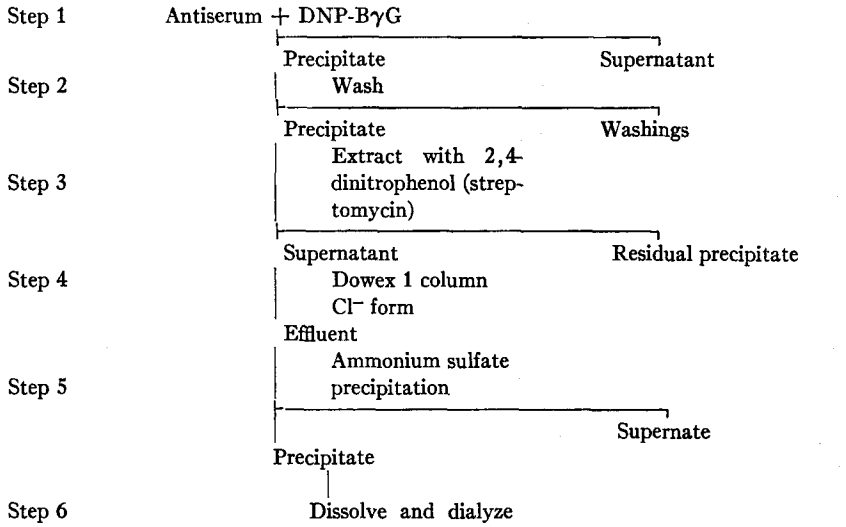

FIG. 1. Scheme for purification of antibodies specific for the 2,4-dinitrophenyl group.

night at 2–5° (step 1). Subsequent steps were carried out at 2–5°, unless specified otherwise. The precipitates were collected by centrifugation, and washed at least twice,[5] each time with 30 ml. 0.15 M NaCl (step 2). The washed precipitates were then finely dispersed in 30 ml. phosphate-saline (0.01 M phosphate, pH 7.4–0.15 M NaCl) containing 2,4-dinitrophenol (0.1 M) and streptomycin sulfate (35 mg./ml.) (step 3). This suspension was incubated for 1 hour at 37°, with frequent gentle stirring, and then centrifuged to yield a supernatant which contained about 50 per cent of the antibody initially precipitated. Longer periods of incubation did not increase the extent of dissociation (see Fig. 4), and dissociation at 2–5° was very much less efficient that at 37°. For example, only about 10 per cent of the antibody in specific precipitates

[5] With larger precipitates, for example those made from 1 to 3 liters of antiserum, washing with 0.15 M NaCl must be carried out repeatedly until the washings are freed of protein as determined qualitatively with trichloracetic acid.

was brought into solution after incubation for 24 hours at 4° in 5×10^{-3} M ε-DNP lysine; the latter hapten has about a 100-fold greater affinity for anti-DNP antibody than 2,4-dinitrophenol (4).

From the available group of haptens, 2,4-dinitrophenol was selected for the extraction of antibody from specific precipitates for three reasons: (*a*) it has a relatively low affinity for anti-DNP antibodies; (*b*) it is highly soluble in water (above pH 7); (*c*) it is easily detected at low concentrations (molecular extinction coefficient of the 2,4-dinitrophenylate ion in water at 360 mμ is 14,600).

The dissociation of precipitates by hapten ordinarily leads to the appearance of antigen as well as of antibody in the soluble phase. The dissolved antigen is objectionable since subsequent removal of haptens leads to losses of antibody through reprecipitation of some of the dissolved antibody (15). In the present procedure the addition of streptomycin to the solution used for dissociation (step 3) minimizes this difficulty by forming insoluble salt-like complexes with the polyanionic antigen (DNP-BγG). BγG extensively substituted with DNP groups is highly anionic since the DNP-substituted ε-amino groups of lysine residues have a markedly reduced p*Ka* (less than 1).[6] After removing insoluble material by centrifugation, the supernatant (about 30 ml.) was passed through an anion exchange column, 36 × 2 cm., that was prepared with about 50 gm. Dowex 1 (× 8, 200 to 400 mesh) in chloride form. The column was washed and subsequently developed with phosphate-saline (0.01 M phosphate, pH 7.4–0.15 M NaCl), the entire procedure (step 4) being carried out at room temperature at a flow rate of 0.25 to 0.50 ml./min. The column effluent was monitored by adding one drop of 1 M perchloric acid to an occasional drop of effluent. In step 5, antibody was precipitated from the effluent by the addition of one volume of saturated ammonium sulfate. By using unbuffered ammonium sulfate (pH ~ 5), antibody was precipitated in cationic form thereby facilitating subsequent removal of streptomycin and ultraviolet absorbing impurities contributed by the resin. The ammonium sulfate precipitate was collected by centrifugation, redissolved in a desired small volume of water, or phosphate-saline, and dialyzed for 24 hours against several changes of 6 liters 0.001 M phosphate, pH 7.4.[7] Small amounts of precipitate which formed during dialysis were discarded.

The final solution was generally lyophilized and stored at about 4°. Under these conditions, antibody activity (see below) was retained indefinitely.

Protein in the final product amounted, generally, to about 40 per cent of that present as antibody in the initial specific precipitate.

Absorption Spectrum of Purified Anti-2,4-Dinitrophenyl Antibodies.—In 0.15 M NaCl–0.01 M phosphate, pH 7.4, purified anti-DNP antibody has an absorption maximum at 278 mμ and a minimum at 251 mμ, the respective extinction coefficients ($E_{1\text{ cm.}}^{1\text{ per cent}}$) being 13.6 and 5.3 (based on micro-Kjeldahl analyses and an assumed N content of 16 per cent; see reference 14). These values, and

[6] Eisen, H. N., unpublished data.

[7] Karush and Marks (15) have shown that rabbit antibodies specific for *p*-azophenyl-β-lactoside and for phenyl-(*p*-azobenzoylamino)-acetate are γ-pseudoglobulins. Rabbit antibodies specific for D-benzyl penicillenic acid are also soluble in 0.001 M phosphate, pH 7–8 (21).

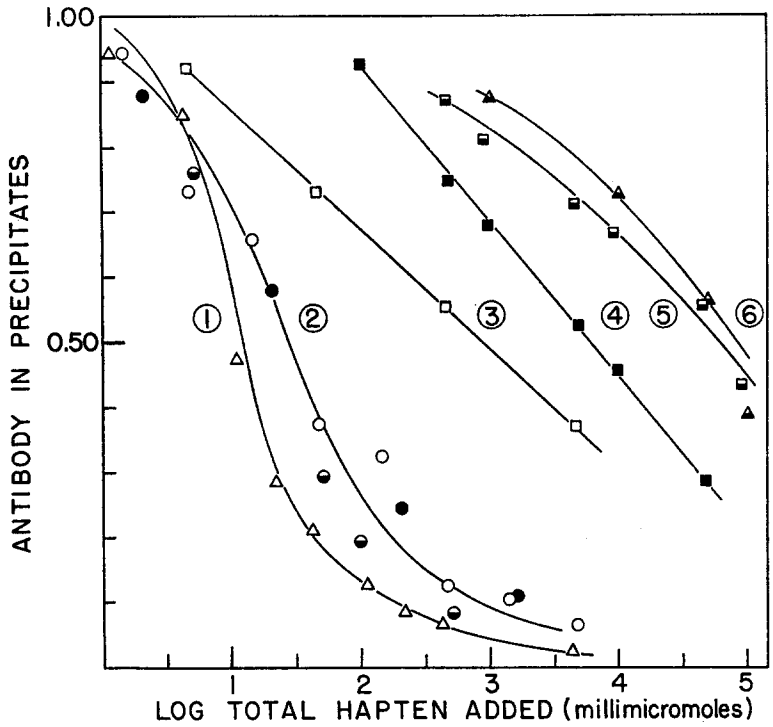

FIG. 2. Inhibition of precipitation and dissolution of specific precipitates, using unfractionated antiserum. Inhibition (curve 1) was carried out by adding varying amounts of ε-DNP lysine to 0.5 ml. of a serum pool. After 15 minutes at ~25°, 125 μg. DNP-Bγ G (46.8 mμeq. DNP) was added (total volume, 1.9 ml.), and incubation was continued for 60 minutes at 37°, and then overnight at 4°. Precipitates were washed and analyzed as described under Methods. In the uninhibited control (zero hapten), the precipitate contained 1.1 mg. antibody and all of the antigen added (46 mμeq. DNP). Four of the points making up curve 1 are average values for precipitates which were made in antibody excess (580 μg. antibody, 47 μg. DNP-Bγ G, or 17.6 mμeq. DNP) or in slight antigen excess (870 μg. antibody, 187 μg. DNP-Bγ G, or 70 mμeq. DNP). As expected, inhibition by a given amount of hapten was more effective with the former system, but the differences were small, and average values are given.

Dissolution was carried out as follows: 0.5 ml. of a serum pool (VI) and 90 μg. DNP-Dγ G (33.6 mμeq. DNP) were incubated for 60 minutes at 37° and overnight at 4°. Precipitates were washed twice with ice cold saline; then resuspended in 1 ml. (0.01 M phosphate–0.15 M NaCl, pH 7.5) containing variable amounts of hapten, and incubated for 60 minutes at 37°. The precipitates were finally again washed twice and analyzed. The control precipitates (unexposed to hapten) contained 870 μg. antibody and 98 per cent of the antigen added (33 mμeq. DNP). In the case of ε-DNP lysine and 3,5-dinitrobenzoic acid, the values given are averages for two sets of precipitates—one made in antibody excess (with 47 μg. DNP-Bγ G), the other in slight antigen excess (with 187 μg. DNP-Bγ G), the respective control precipitates (zero hapten) containing 578 μg. antibody and all the antigen added (17.9 mμeq. DNP), and 870 μg. antibody with 176 μg. DNP-Bγ G (66 mμeq. DNP).

The amount of antibody in precipitates is given on the ordinate as a fraction of the amount in control precipitates (zero hapten).

Curve 1, inhibition by ε-DNP lysine (△). Curve 2, dissolution by ε-DNP lysine (●), ε-DNP aminocaproate (○), and DNP-lysyl peptides (◒). Curve 3, dissolution by DNP-glycine (□). Curve 4, dissolution by DNP-acetic acid (■). Curve 5, dissolution by dinitrophenol (◪). Curve 6, dissolution by 3,5-dinitrobenzoic acid (▲).

the complete absorption spectrum obtained, are in close agreement with those found for purified rabbit antibodies specific for the phenyl-(*p*-azobenzoyl-amino)-acetate group (15). Similar spectral values have also been found for rabbit antibodies in the form of specific precipitates dissolved in dilute acetic acid (13, 16). While the ratio of absorbancies at $\lambda_{max.}/\lambda_{min.}$ has only limited usefulness in establishing the purity of purified antibodies, these ratios can, at least, serve to reveal the presence of ultraviolet-absorbing impurities. The ratios of absorbancies at 278 mμ/251 mμ for many different preparations of purified anti-DNP antibodies were nearly always in the range 2.5 to 2.75 (*cf.* reference 15).

Specific Precipitability of Purified Antibody.—Solutions of purified antibody did not precipitate on addition of BγG. With DNP-BγG, precipitation was prompt and nearly complete. The extent of precipitation was determined quantitatively by analyses of washed precipitates and also by direct absorbance determinations of the supernatants; the latter procedure is more rapid and direct. Representative data for precipitate and supernate analyses of a precipitin reaction performed with purified antibody are given in Table I. With five different preparations, from different lots of pooled antisera, specific precipitability of the purified protein, at the point of maximum precipitation, varied from 86 to 92 per cent.[8]

If some of the dinitrophenol used for extraction of antibody from specific precipitates persisted through subsequent purification steps, precipitation of the final product by antigen (DNP-BγG) would, of course, be inhibited. Solutions of purified antibody were colorless, but from the molecular extinction coefficient of dinitrophenol, and from the data of Fig. 2, it is apparent that inhibitory concentrations could be present and not be detected spectrophotometrically. To examine this possibility, a small scale purification was carried out with 1-C^{14}-2,4-dinitrophenol and the final product (purified antibody) was analyzed for C^{14}. In 1 ml. containing 1.8 mg. antibody, 327 C.P.M. were found, corresponding to 2.4 mμmoles of dinitrophenol. Since the purified antibody has a molecular weight of 160,000 and two sites per molecule, about 10 per cent of the antibody-binding sites were still occupied by dinitrophenol. These persistent traces of hapten account, at least in part, for the fact that precipitability of the purified antibody was less than 100 per cent.

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[8] Precipitability of purified antibody preparations was generally determined in 1.0 ml. total volume containing about 1.0 mg. purified protein and varying amounts of DNP-BγG. C. W. Parker (unpublished data) has noted that the extent of precipitability is less at lower concentrations of the purified antibody.

Specific precipitation takes place far more rapidly in solutions of purified antibody than in whole antiserum. For example, C. W. Parker (unpublished observations) has noted that precipitation after only 30 minutes at 37° is 86 per cent as great as is found after 60 minutes at 37° followed by 1 to 5 days at 4°; precipitation is no greater after 5 days than after 1 day in the cold.

Dissolution of Specific Precipitates and Inhibition of Precipitate Formation by Haptens.—In the purification procedure described above, antibodies were not extracted completely from specific precipitates by 2,4-dinitrophenol. As a consequence, it may be expected that the antibodies finally isolated could, on

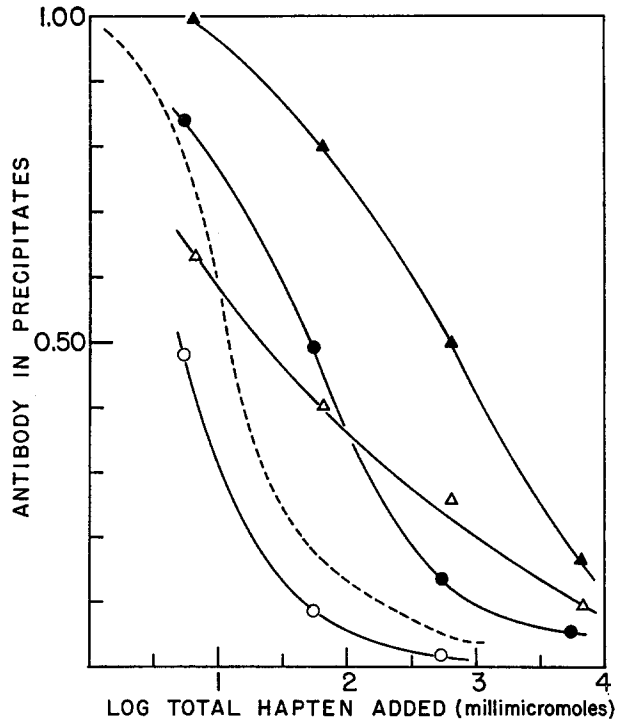

FIG. 3. Inhibition of precipitation and dissolution of specific precipitates, using purified antibody. Inhibition was carried out as described in the legend of Fig. 2 except that purified antibody was used in place of antiserum. The uninhibited control (zero hapten) had 736 μg. antibody and 93.6 μg. DNP-BγG (35 mμeq. DNP). Dissolution was performed as described in the legend of Fig. 2 except that the specific precipitate was prepared from purified antibody. The control (unexposed to hapten) had 354 μg. antibody and 83 μg. DNP-BγG (31 mμeq. (DNP). Ordinate is the same as in Fig. 2. Symbols are as follows: (○) ε-DNP lysine inhibition; △) 2,4-dinitrophenol inhibition; (●) ε-lysine dissolution; (▲) 2,4-dinitrophenol dissolution.

Curve 1 of Fig. 2 (inhibition of precipitation in unfractionated antiserum by ε-DNP lysine) is repeated (broken line) for purposes of comparison.

the average, have a relatively greater affinity for this hapten than the unfractionated population of antibodies present in the original antisera. This possibility could not be evaluated by comparing dinitrophenol inhibition of precipitation of purified antibody and of whole antiserum since dinitrophenol is strongly bound by serum albumin (5). Instead, the ability of this hapten to dissolve specific precipitates, made with DNP-BγG and either unfractionated serum or

purified antibodies, was examined. For purposes of further comparison, other dinitrophenyl haptens were compared in respect to capabilities for dissolving these two types of specific precipitates. In the case of purified antibody, 2,4-dinitrophenol was also examined for its ability to inhibit precipitation, and ε-DNP lysine, which is essentially not bound by serum albumin (5), was

TABLE II

*Comparison of Unfractionated Antiserum and Purified Antibody in Respect to Amounts of Haptens Required for 50 Per Cent Inhibition of Precipitation and for 50 Per Cent Dissolution of Specific Precipitates**

Haptens	Precipitates made with			
	Antiserum		Purified antibody	
	Total hapten needed	Total antigen in control precipitate (zero hapten), as DNP‡	Total hapten needed	Total antigen in control precipitate (zero hapten), as DNP‡
	mμmoles	*mμeq.*	*mμmoles*	*mμeq.*
For inhibition				
ε-DNP lysine	13	18–70	5	35
2,4-Dinitrophenol			22	35
For dissolution				
ε-DNP lysine	30	18–66	50	31
ε-DNP aminocaproate	30	33		
ε-DNP lysyl peptides	30	33		
DNP glycine	900	33		
2,4-Dinitrophenylacetate	6,000	33		
2,4-Dinitrophenol	60,000	33	630	31
3,5-Dinitrobenzoate	83,000	18–66		

* For conditions used to obtain these data see legends of Figs. 2 and 3.

‡ Based on 360 mμ absorbance of dissolved specific precipitates. A solution of DNP-BγG is assumed to have 10 mμeq. DNP per ml. when its absorbance at 360 mμ is 0.174. See legends of Figs. 2 and 3 for additional details concerning procedures and control precipitates (*i.e.* zero hapten). It is probable that only about 30 per cent of the DNP groups of the antigen (DNP-BγG) can react at the same time with antibody (4); presumably the rest are sterically inaccessible.

examined for its ability to inhibit precipitation in whole serum. Procedural details of these experiments are given in Figs. 2 and 3 and the results are summarized in Table II. Except for the fact that a precipitate is present at zero time, hapten dissolution is, in principle, no different from hapten inhibition of precipitation. Accordingly, the data obtained may be treated as described by Pauling and Pressman (17). From the inhibitory effect of ε-DNP lysine (curve 1, Fig. 2), it appears that the anti-DNP antibodies in an unfractionated pool of

anti-DNP sera are relatively homogeneous in respect to their intrinsic binding affinities for this hapten (heterogeneity index, σ, is about 1.5).

Hapten dissolution of precipitates involves competition for antibodies between hapten and the corresponding determinants of antigen (DNP-BγG). Hence, the greater effectiveness of 2,4-dinitrophenol in dissolving specific precipitates made with purified antibody (Table II) must mean that the latter have either a greater affinity for this hapten, or a lower affinity for DNP-BγG (or both), than the average antibody in unfractionated serum. Quantitative

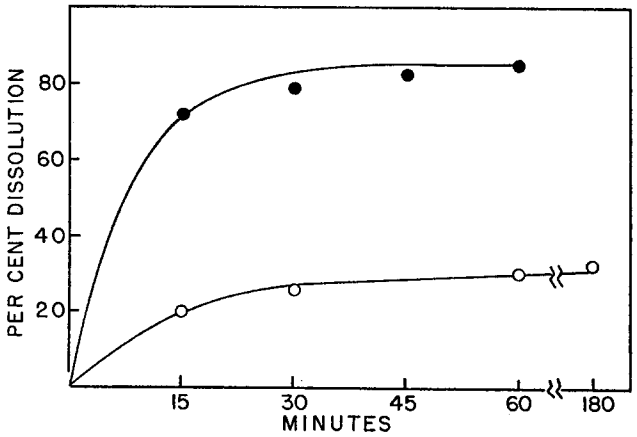

FIG. 4. Time course for hapten dissolution of specific precipitates. Washed specific precipitates were incubated at 37° for the periods shown with 1.0 ml. of 1×10^{-3} M (●) or 1.0 ml. of 1×10^{-5} M (○) ϵ-DNP lysine. The precipitates were then washed twice with cold 0.15 M NaCl, air-dried, dissolved in 0.25 M acetic acid, and analyzed spectrophotometrically.

discrimination between these several possibilities can be made only when the residual antibody fraction, not extracted with dinitrophenol, becomes available in purified form. For the present, however, it may be noted that about 1000 times more dinitrophenol than ϵ-DNP lysine is needed to dissolve 50 per cent of a standard specific precipitate made from unfractionated antiserum, but that only about 10 times more dinitrophenol than ϵ-DNP lysine is required to dissolve 50 per cent of a specific precipitate made from purified antibody. It appears, therefore, that the present purification scheme selects that fraction of total anti-DNP antibody with a relatively high affinity for 2,4-dinitrophenol.

Since the association of antibody with antigen, and with hapten, is reversible, a given mixture of these three reagents should, if allowed to reach equilibrium, give the same amount of precipitate regardless of whether the hapten is added to a preformed precipitate (hapten dissolution) or is added to antibody prior to the addition of antigen (hapten inhibition). Actually, however, slightly greater amounts of ϵ-DNP lysine were needed to dissolve precipitates than to

inhibit their formation (Figs. 2, 3, and Table II), probably because of inhomogeneity in the dissolution experiments. Nevertheless, the time course of dissolution (Fig. 4) demonstrates that the conditions used were adequate for our purpose.

DISCUSSION

Antisera with relatively high concentrations of antibody (*e.g.*, more than 1 mg. antibody/ml.) are desirable for the development of procedures for purifying antibodies. In the case of anti-DNP sera, this requirement is met by injecting rabbits with Freund's complete adjuvant (10) containing protein that is just about maximally substituted with DNP groups.[9] These conditions are contrary to certain prevalent opinions, *i.e.* (*a*) that the use of Freund's adjuvant with protein-hapten conjugates is inadvisable for procurement of potent anti-hapten sera as it supposedly favors the production of anti-protein antibodies rather than anti-hapten antibodies (18), and (*b*) that highly substituted proteins are less effective than those with a lesser degree of substitution. The latter view, which has been interpreted as having significance for an "instructive" hypothesis of antibody formation (19), seems to stem mostly from Haurowitz's work with benzene arsonate-azoproteins (20). In that work Haurowitz observed that azoglobulin antigens with more than 3 per cent arsenic were less effective inducers of anti-azobenzene–arsonate antibodies than were antigens with 1 to 2 per cent arsenic. The latter conjugates were supposed to have 10 to 20 azo determinants per molecule of globulin, but actually had about 20 to 40 azo substituents and were, therefore, not as lightly substituted as has generally been supposed. The benefits of Freund's adjuvant and highly substituted conjugates in producing anti-hapten antibodies are not confined to the 2,4-dinitrophenyl determinant. The same conditions have been useful also in procuring potent antisera specific for D-benzylpenicillenic acid (21).

Since the classic studies by Heidelberger and his associates on the purification and characterization of antibodies specific for pneumococcal polysaccharides (22), a number of methods have been developed for the preparation of purified antibodies (see references 23–25 for recent reviews).[10] The present method resembles some others (*e.g.*, reference 15) in basic principles; *i.e*, it depends on the formation of a specific precipitate, the extraction of antibodies from the

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[9] In earlier work, by contrast, the antisera prepared by sixteen intravenous injections over a 4 week period of alum-precipitated DNP-BγG (25 mg. protein; about 10 moles DNP/mole protein) had much lower concentrations of anti-DNP antibodies and a large fraction of the total antibody produced was precipitated with BγG (12).

[10] Singer *et al.* (27) have very recently described a novel procedure for the purification of antibodies specific for protein antigens. Their interesting experiments, which depend on the preservation of antigenic function in thiolated proteins, promise to make available a fairly general method for the preparation of highly purified anti-protein antibodies.

precipitate with a hapten, and fractionation of soluble hapten-antibody complexes leading to isolation of purified antibodies essentially free of hapten. Three features of the present method, however, require discussion. These are: (*a*) the choice of hapten for extraction of antibody from precipitates, (*b*) the difficulties encountered in separating hapten from soluble hapten-antibody complexes, and (*c*) the usefulness of streptomycin.

Rabbit antibodies are remarkably tolerant of wide variations of ionic strength, pH, urea concentration, etc. (see, for example, reference 4). Nevertheless, the possibility cannot be neglected that use of extreme conditions for dissociation of specific precipitates or soluble antibody-hapten complexes may cause irreversible structural modifications of an antibody without necessarily altering its specific binding sites. In the present method, as in that of Karush and Marks (15) and others, the use of haptens permits dissociation of antibody from the initial antigen-antibody specific precipitate under such gentle conditions (*i.e.* 37°, pH 7.5, 0.15 M NaCl) that structural changes ought to be at a minimum. Haptens of high affinity would seem to be the obvious choice for extraction purposes as they can remove antibody completely, or nearly so, from specific precipitates. Subsequent dissociation of such haptens from soluble antibody complexes is, however, likely to be difficult. For example, ε-DNP lysine and ε-DNP aminocaproic acid extract virtually all anti-DNP antibody from specific precipitates, but it has been impossible, so far, to separate these haptens from the corresponding soluble antibody complexes by a variety of procedures, including such obvious ones as dialysis and ion exchange columns. A hapten of low affinity offers a satisfactory compromise, provided that its solubility is sufficiently great; *e.g.*, 2,4-dinitrophenol in the present system. It is important to note, however, that a weak hapten of this type solubilizes only a fraction of the precipitated antibody, and that this fraction is probably selected because it has, on the average, a higher affinity for dinitrophenol. As a consequence, separating even dinitrophenol from its soluble complex with antibody was more difficult than had been anticipated. Prolonged dialysis was not adequate. It was necessary to resort to an anion exchange column, and even with resin treatment followed by ammonium sulfate precipitation and dialysis, it was possible to demonstrate (with C^{14}-labeled hapten) persistence of traces of dinitrophenol: about 10 per cent of the specific binding sites in the final product were still occupied by this hapten.

When antibodies are extracted from specific precipitates, some antigen also becomes soluble. This is certainly the case when the extraction is specific (*i.e.* with haptens) and is probably equally true when non-specific means are used (*e.g.* low pH). Streptomycin minimizes antigen solubility during extraction in the present procedure presumably because it forms insoluble salt complexes with proteins that are highly anionic as a consequence of extensive substitution with DNP groups. It is possible that the use of streptomycin can be extended

to other antigen-antibody systems in which the antigen, without altering its determinants, can be made more anionic by appropriate substitutions of lysine ϵ-NH_2 groups, *e.g.* with acetyl, or even dinitrophenyl groups. This principle has been successfully applied to the isolation of purified antibodies specific for the azobenzene-arsonate group.[11]

The dissolution of specific precipitates by a number of different dinitrophenyl haptens was examined systematically in the present work primarily to select the most suitable specific reagent for the extraction of antibodies from precipitates. This procedure (hapten dissolution) provides also a simple means for estimating crudely, and in relative terms, antibody-hapten affinities (in competition with antigen); it may offer some advantages over hapten inhibition of precipitation in cases in which non-specific binding of haptens by serum albumin or other serum proteins is troublesome. The haptens examined fell in the following order in respect to their capacities to dissolve specific precipitates: ϵ-DNP lysine = ϵ-DNP aminocaproate = DNP–lysyl peptides > DNP-glycine > dinitrophenyl-acetate > dinitrophenol $\cong$ 3,5-dinitrobenzoate. The fact that ϵ-DNP lysine and ϵ-DNP aminocaproate have essentially equal affinities indicates that charged groups, remote from the DNP nucleus, make little, if any, contribution to the antibody-hapten interaction. This finding is consistent with the fact that in the immunizing antigen, DNP-lysine residues are in α-peptide bond. The lesser effectiveness of DNP-glycine and the still smaller effect of DNP-acetic acid undoubtedly arise from their lacking, respectively, the 3 methylene groups and the substituted amino N of ϵ-DNP lysine and ϵ-DNP aminocaproate. A mixture of DNP-lysyl peptides, prepared from the immunizing antigen (DNP-BγG), is of interest in view of the evidence that the combining sites of antibodies may be complementary for relatively large structures; *i.e.*, an oligosaccharide containing 6 hexose residues in the case of anti-dextran antibodies (26). Per mole DNP, however, these mixed DNP-lysyl peptides were hardly more effective, if at all, than ϵ-DNP lysine (Fig. 2). These findings suggest that the effective binding region of purified rabbit anti–DNP antibody is probably not much larger, if at all, than is required to accommodate ϵ-DNP lysine.

SUMMARY

Injection of rabbits with Freund's complete adjuvant containing bovine γ-globulin substituted to near saturation with dinitrophenyl groups leads to the production of large amounts of antibodies specific for the 2,4-dinitrophenyl substituent. These antibodies can be isolated from sera in satisfactory yield (40 per cent) and in a high state of purity (90 per cent specifically precipitable). Hapten dissolution, which was used to screen haptens for their potential useful-

[11] Parker, C. W., Kern, M., and Eisen, H. N., unpublished results.

ness as specific reagents in the purification procedure, provides a means for evaluating, in relative terms, antibody-hapten affinities (in competition with antigen).

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