STUDIES ON THE DIMENSIONS OF THE RABBIT ANTI-BENZYLPENICILLOYL ANTIBODY-COMBINING SITES*,‡

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The dimensions of the specific combining regions of the antibody molecule (*i.e.*, the combining sites) is of fundamental importance for an understanding of the mechanisms of antibody-antigen interaction, and the biological effects of these interactions. The work of Kabat (1) which utilized quantitative hapten inhibition methods, indicates that human anti-dextran antibody combining sites are sufficiently large to encompass an antigenic unit the size of a hexasaccharide. Similarly, Cebra's studies (2) indicates that rabbit anti-silk fibroin antibody-combining sites are complementary to an antigenic unit at least the size of an octapeptide or a dodecapeptide. Several other works which examined rabbit anti-hapten antibodies by quantitative precipitation methods indicated that at least some of the anti-hapten antibody molecules possessed combining sites complementary to an antigenic unit comprised of hapten and adjoining carrier protein structures. (Haurowitz, *p*-azoarsanilic acid (3); Eisen *et al.*, dinitrophenyl (4); and Buchanan-Davidson *et al.*, synthetic polypeptides (5)).

In sharp contrast to these findings, Landsteiner and van der Scheer (6) using qualitative absorption and precipitation methods, concluded that rabbit antibodies to an azostroma in which the hapten consisted of an arsanilic acid group and a succinanilic acid group attached to the same benzene ring, (*i.e.* a bifunctional hapten, Fig. 1, SA hapten) were complementary to one group or the other, but were not complementary to the entire bifunctional hapten. They obtained similar results with a second bifunctional hapten, azoisophthalyl-glycine-leucine (Fig. 1, GIL hapten). These results would indicate that, at least for these systems, the antibody-combining sites were not sufficiently large to encompass the entire bifunctional hapten molecule.

Recently, the benzylpenicilloyl-lysine haptenic group (Fig. 1) which results from the reaction of D-benzylpenicillenic acid with lysine ϵ-NH_2 groups of protein, has become available for study (7). The BPO-lysine[1] group is a comparatively large

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[1] Abbreviations: BPO, benzylpenicilloyl group; AMMPO, allylmercaptomethylpenicilloyl group; DMPPO, dimethoxyphenylpenicilloyl group; PAC, phenylacetylamine group Proteins are abbreviated: HGG, human γ-globulin; HSA, human serum albumin; RGG, rabbit γ-globu-

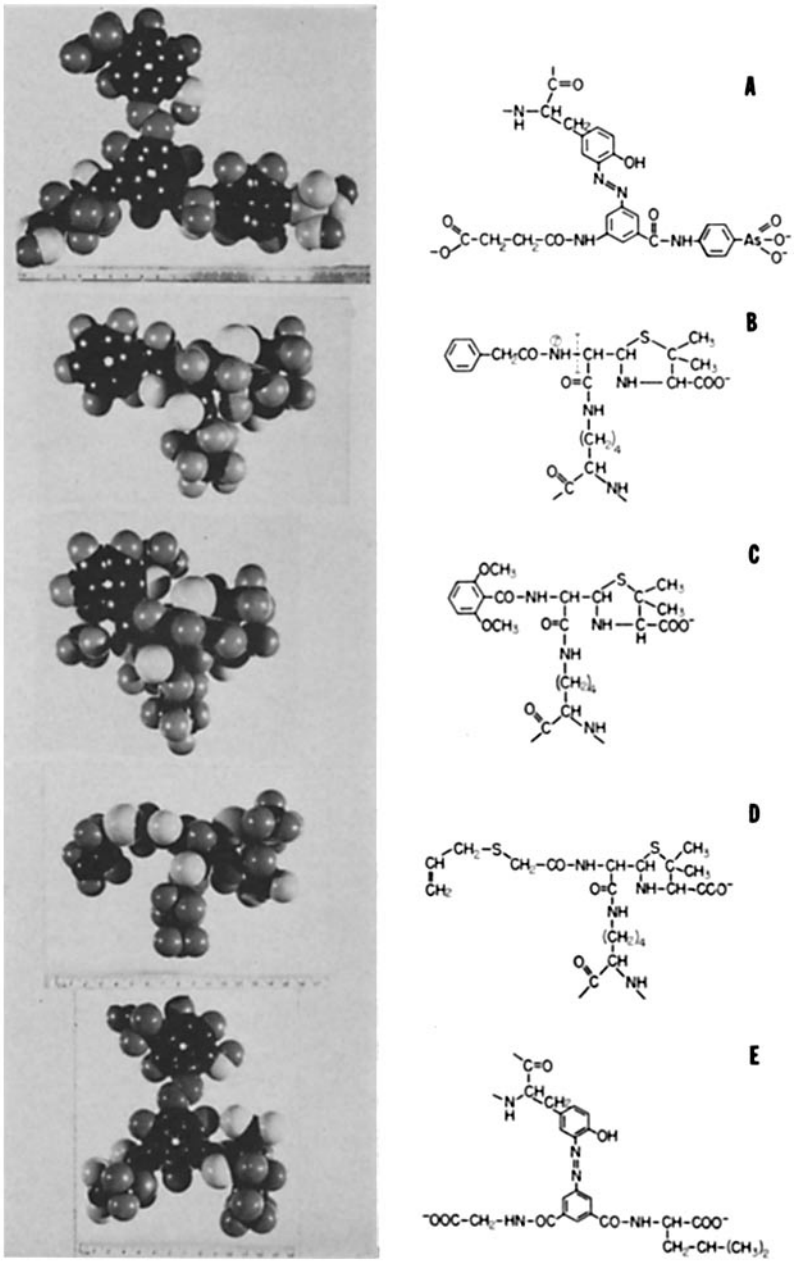

FIG. 1. Structural formulas and photographs of Fisher-Hirschfelder-Taylor scale molecular models of several bifunctional haptenic groups. 1 cm. = 1 Å, *A*, SA haptenic group; *B*, BPO group; *C*, DMPPO group; *D*, AMMPO group; and *E*, GIL group. The SA and GIL haptenic groups are shown coupled to tyrosine residues of protein; the penicilloyl haptenic groups are shown coupled to lysine ϵ-NH_2 residues of protein. The BPO group is shown divided by brackets into the phenylacetylamine part (left) and the thiazolidine carboxylic acid part (right).

haptenic group, intermediate in size between the two bifunctional haptens used by Landsteiner (Fig. 1). The BPO group also may be considered a bifunctional hapten comprised of a non-polar phenylacetylamine end and a polar thiazolidine carboxylic acid end (Fig. 1). The BPO group is of additional interest since it has been demonstrated to be the major antigenic determinant responsible for hypersensitivity to benzylpenicillin in human beings and in experimental animals (7, 8).

In the present work rabbit antisera prepared against the BPO-lysine haptenic group were examined to determine whether anti-BPO antibody-combining sites are adapted to only the phenylacetylamine end or to the thiazolidine carboxylic acid end of the BPO molecule, or whether the combining sites encompass the entire BPO haptenic group. Also investigated was the extent to which anti-BPO antibody combining sites are directed against the lysine side chain (through which the BPO groups are bound predominantly to the protein carrier), and whether the anti-BPO combining sites are adapted also to structural areas of the carrier protein.

The results obtained demonstrate that at least a large part of rabbit anti-BPO antibodies are specifically adapted to an antigenic unit comprised of the entire BPO group, the lysine side chain, and structúral areas (probably adjoining the point of attachment of the hapten) of the carrier protein. No antibodies which encompass only the phenylacetylamine end or the thiazolidine carboxylic acid end of the BPO molecule were detected in the rabbit anti-BPO sera.

Experimental

Materials.—Crystalline potassium benzylpenicillin (Lot F-0079) and sodium dimethoxyphenylpenicillin (staphcillin, Lot A-1028) were donated by Bristol Laboratories,[2] Syracuse, New York. Crystalline sodium allylmercaptomethylpenicillin (Cer-O-Cillin, Lot MM-344) was donated by the Upjohn Co.,[2] Kalamazoo, and crystalline sodium benzylpenicillin was donated by Pfizer Laboratories,[2] Brooklyn. Other chemicals were of reagent grade. Complete Freund's adjuvant was a product of Difco Laboratories, Detroit.

Poly-L-Lysine·HCl with an average chain length of 20 units (manufacturer's analysis, Lot 02202) was donated by the Kremers-Urban Co.,[2] Milwaukee. Human serum albumin, and human γ-globulin were gifts of the American Red Cross. RSA[1] was prepared from pooled normal rabbit serum by ammonium sulfate fractionation. RSA was purified further by a method similar to the trichloracetic acid method described by Schwert for bovine serum albumin (9), which is reported to result in little or no protein denaturation (9, 10). By paper

lin; RSA, rabbit serum albumin; PLL, poly-L-lysine. Conjugates are abbreviated: BPO_{22}-HGG, $DMPPO_{27}$-RSA *etc.* for benzylpenicilloyl- human γ-globulin and dimethoxyphenylpenicilloyl-rabbit serum albumin. Subscript numbers refer to the average number of haptenic groups per molecule of conjugate. Univalent penicilloyl amine haptens are abbreviated: BPO-EACA, for benzylpenicilloyl-ϵ-aminocaproate; DMPPO-*n*-propylamine for dimethoxyphenylpenicilloyl-*n*-propylamine *etc.* B.S., buffered saline, 0.15 M sodium chloride, 0.02 M phosphate.

[2] We thank Drs. J. Doyle of Bristol Laboratories., J. O. Lawrence of Upjohn Co., W. Fuhrman of Pfizer Labs., B. Lees of Kremers-Urban Co., and J. H. Pert of the American Red Cross for gifts of these materials.

electrophoresis and by immunoelectrophoresis,[3] the material was pure except for a trace of α_2-globulin. Protein contents of these preparations were determined by duplicate micro-Kjeldahl (11 *a*) analyses.

Ultraviolet absorption studies were done with a Zeiss model PMQ 11 spectrophotometer and 1 cm light path matched quartz cuvettes. Optical rotations were taken with a Schmidt and Haensch polarimeter using a 2 decimeter cell.

Penicillenic Acids.—D-Benzylpenicillenic acid was prepared by method described previously (12): ϵ = 24,000 at λ_{max} 322 mμ. D-Allylmercaptomethylpenicillenic acid was prepared from sodium allylmercaptomethylpenicillenic by the method used for D-benzylpenicillenic acid; λ_{max} 328 mμ, ϵ = 11,000,[4] (95 per cent ethanol). D-Dimethoxyphenylpenicillenic acid was prepared as follows:[5] To a solution of 7.0 gm. (16.7 mmoles) of sodium dimethoxyphenylpenicillin and 4.55 gm. (20.2 mmoles) mercuric chloride in 250 ml water at room temperature, was added dropwise with stirring 16.7 ml of 1.0 N HCl.[4] Stirring was continued for 1 hour. The precipitated yellow mercaptide was gathered, washed with water, and converted to free dimethoxyphenylpenicillenic acid by the procedure used for benzylpenicillenic acid; Y = 2.4 gm (37 per cent) of yellow powder; λ_{max} 335 mμ, ϵ = 24,300 (95 per cent ethanol). Purity of benzylpenicillenic acid was judged to be 78 per cent based on molar extinction coefficient and correcting for 13 per cent unreactive penicillenic acid disulfide present in the preparation (12). Assays of dimethoxyphenylpenicillenic acid and allylmercaptomethylpenicillenic acid were estimated to be 75 per cent and 50 per cent, respectively. Penicillenic acid preparations were stored in calcium chloride desiccators at −10° and were stable for at least 6 months.

Preparation of Penicilloyl-Protein Conjugates.—A solution of the penicillenic acid in 95 per cent ethanol was added dropwise to the gently stirred 1 per cent solution of the protein in 0.1 M phosphate buffer, pH 8, at room temperature. Total ethanol was generally 5 to 10 per cent of the reaction volume. pH was maintained at 7.5–8.2 by addition of 1 M potassium carbonate either manually or from a pH stat. Generally, from 0.3 to 2.5 molar equivalents of the penicillenic acid per mmole of lysine ϵ-NH_2 group contained in the protein was used. Reactions were complete within 20 minutes after penicillenic acid was added. After an additional 10 minutes of stirring, the reaction solution was incubated at 37° for 1 hour with cysteine (0.1 M concentration, pH 7.5) in order to reduce off penicillenic acid disulfide and other disulfide groups. The conjugates were freed of low molecular weight side products by four 24 hour dialyses at 4° against 2 gm amberlite IRA-400, (400 to 600 mesh) resin (previously equilibrated with tris buffer, pH 8.5) suspended in 0.002 M tris (tris-(hydroxmethyl)-aminomethane) buffer, pH 8.0. After a final dialysis against 0.005 M phosphate buffer, pH 8.0, the conjugates were stored without preservative at −10°C where they were stable for at least 6 months. Additional procedural details are given in reference 12.

Preparation of Penicilloyl-Polylysine Conjugates.—To 240 mg of PLL·HCl (1.45 mmoles ϵ-NH_2) dissolved in 15 ml of 0.25 M potassium bicarbonate, was added dropwise with stirring a solution of 0.66 mmoles of the penicillenic acid dissolved in 1.5 ml of 95 per cent ethanol. The stirring was continued for 30 minutes and pH was maintained at 8.0–8.5 by additions of 1 M potassium carbonate. The precipitated penicilloyl-polylysine was centrifuged off, washed, suspended in 10 ml of 0.1 M carbonate buffer, and reacted at room temperature, pH 10, with

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[3] Performed by Dr. G. M. Hochwald of New York University Medical School.

[4] D-Allylmercaptomethylpenicillenic acid appears to be hydrolized more readily than is D-benzylpenicillenic acid. The penicillenic acid rearrangement is much slower for dimethoxyphenylpenicillin than it is for the other 2 penicillins. It requires a lower pH for the rearrangement to go to completion at room temperature and within a reasonable period of time.

[5] The preparation of dimethoxyphenylpenicillenic acid was carried out with Dr. R. Schwartz of The University of Rochester Medical School.

2.0 mmoles succinic anhydride.[6] The filtered reaction solution was adjusted to pH 1.8 with 1 N HCl. The precipitated penicilloyl-PLL (succinylated) was gathered and washed thoroughly with H_2O, ethanol, and then ether. Y = 330 mg of white powder. The powder was dissolved in buffered saline (0.15 M sodium chloride, 0.02 M phosphate, pH 8), with additions of 1 M NaOH and the solution was stored at −10°C, where it was stable for at least 3 months.

Preparation of Phenylacetyl-Protein Conjugates.—To a stirred 1 per cent solution of the protein in 0.1 M pH 8 phosphate buffer at room temperature was added dropwise a solution of phenlyacetyl chloride in dioxane. Generally 1 to 3 molar equivalents phenylacetyl chloride per mmole ϵ-NH_2 contained in the protein was used. Total dioxane was 5 per cent of the reaction volume. pH was maintained between 7.5–8.5 with 1 M potassium carbonate and the reaction was complete within 10 minutes. Precipitated material was centrifuged off and the conjugate was purified by repeated dialyses as described above.

Analyses of the Conjugates.—Protein assays were done by duplicate micro-Kjeldahl analyses (11*a*) and corrected for the nitrogen contribution of penicilloyl groups; mean deviation ± 3 per cent. Assay for penicilloyl groups was done by penamaldate method as described previously (7); ϵ at λ 285 mμ for BPO, 23,800; AMMPO, 23,900; DMPPO, 20,500. Duplicate analyses were done, mean deviation, ± 3 per cent. Free lysine ϵ-NH_2 groups were assayed by formol titration (13), pH_i, 8.5; pH_f, 9.2, with correction for formol blanks; mean deviation for duplicate analyses, ± 5 per cent.

Univalent Haptens.—Penicilloyl amines were prepared as their crystalline benzylammonium salts, α-diastereoisomers, and recrystallized several times to constant, sharp melting points. The steric equilibrium mixtures of diastereoisomers of the penicilloyl amines were used in hapten inhibition experiments. They were prepared from the α-diastereoisomers by refluxing in aqueous solution pH 5, for a period of time sufficient to allow partial racemization to reach equilibrium. The resulting solutions contained 98 to 99 per cent of the penicilloyl amines and 1 to 2 per cent penamaldoyl amines which were formed by rearrangement of penicilloyl amines during reflux. Details of these preparations, elemental analyses, physical and chemical properties of the penicilloyl amines have been given previously (14). Penicilloyl amine solutions were assayed immediately before use in hapten inhibition experiments by the penamaldate method as described previously (14). For analysis of AMMPO-EACA, the hapten was diluted in a pH 9.2 carbonate buffer solution containing 1 mg per ml HSA which served to stabilize the 285 mμ peak, ϵ = 23,900 ± 1 per cent.

Monosodium D-α-benzylpenicilloate: ½ H_2O, α-diastereoisomer, was prepared as described previously (7), m.p., 154–155° (micro). Its equilibrium diastereoisomeric mixture was prepared by incubation of a 2.0×10^{-2} M solution of the α-diastereoisomer in B.S.,[1] pH 7.5, 37° for 6 hours; $[\alpha]_D^{25}$ for α-diastereoisomer, + 130°; for equilibrium diastereoisomeric mixture, + 35.8°

N-Phenylacetylglycine was prepared by reaction of 1 molar equivalent of phenylacetyl chloride with 1.2 equivalents of glycine in aqueous solution at room temperature, pH 11.5. Phenylacetylglycine was precipitated from the reaction mixture at pH 1.8 and crystallized twice from hot water. Y = 50 per cent, white plates; m.p., 144–145°; reported (15), 142–144°.

Antisera.—White rabbits, 4 kg, of either sex were used. Bleedings were from the ear arteries (16). Sera were stored without preservative at −10°.

Pooled Rabbit Anti-Benzylpenicilloyl Serum, Globulin Fraction.—Six rabbits were immunized with a preincubated mixture of potassium benzylpenicillin and pooled normal rabbit serum emulsified in complete Freund's adjuvant. The immunization and bleeding schedules have

[6] Under these conditions, succinic anhydride reacts readily with the ϵ-NH_2 groups of polylysine, but does not react appreciably with the thiazolidine secondary amine of the BPO molecule (unpublished data).

been described previously (7). The serum from the 6 animals were pooled, and the globulin fraction from 260 ml serum was separated by precipitation at room temperature with ammonium sulfate, at a final concentration of 1.75 M, pH 7.2. The precipitated globulin was washed thoroughly with 1.75 M ammonium sulfate. It was suspended in water, the suspension dialysed extensively against B.S.[1] pH 7.3, and finally dissolved in a volume of 200 ml. The product was free of albumin by paper electrophoretic and immunoelectrophoretic analyses.[3]

Rabbit anti-(BPO_n-RSA), Sera 4 and 5.—Two rabbits were immunized by the following schedule: Day 1: 5 mg antigen dissolved in 1 ml B.S. and emulsified with 1 ml complete Freund's adjuvant was distributed into 4 dorsal subcutaneous sites. Day 30: The same dosage was distributed into 8 dorsal intradermal sites. Days 61, 62, 63: 2 mg antigen was injected intravenously. Days 70, 71: Animals were bled and the serums from the two bleedings were combined for each animal.

Rabbit anti-(BPO_4-RSA), Serum 34.—One rabbit was immunized by the following schedule: Day 1: 5 mg antigen dissolved in 1 ml B.S. and emulsified with 1 ml complete Freund's adjuvant was distributed into 8 dorsal intradermal sites. Days 30, 31: 2 mg antigen was injected intraperitoneally twice daily. Days 37, 38, 39: Animal was bled and the sera from the bleedings were combined.

Decomplementation of Sera.—A washed ovalbumin–rabbit antiovalbumin precipitate prepared at equivalence was finely suspended in the serum. The suspension was incubated at 37° for 1 hour, and at 4° for 2 days with intermittent resuspension, and the precipitate was removed by centrifugation. 100 μg precipitate N per ml serum was used as recommended by Maurer and Talmadge (17). Quantitative precipitin analyses (11 *b*) of the antisera before and after decomplementation showed a decrease of 60 to 90 μg protein per ml precipitated at equivalence, consistent with reported experience (17). A second decomplementation did not reduce further the weight of antibodies precipitated at equivalence.

Quantitative Precipitin Analyses of Antisera.—The methods developed by Heidelberger were used (11 *b*). Antisera were clarified by repeated centrifugation at 12,000 RPM. To 0.50 ml aliquots of clarified antiserum or globulin fraction was added increasing amounts of antigen dissolved in 0.50 B.S.,[1] pH 7.3. Reaction mixtures as well as antigen (at various quantities) and antiserum controls were set up in duplicate and incubated at 37° for 1 hour, and at 4° for 68 hours with intermittent resuspension of the precipitates. Preliminary experiments showed that maximum precipitation was achieved under these conditions in all systems studied here. Precipitates were washed 3 times with 2.0 ml of ice cold saline, air-dried, dissolved in 0.1 N sodium hydroxide, and measured aliquots were analyzed by the Folin method (18); mean deviation in O.D. at λ_{max}, 750 mμ ± 3 per cent. The Folin method was standardized against Kjeldahl-analyzed solutions of RGG[1] (Fx II, Pentex Inc., Kankakee, Illinois), several penicilloyl-HGG preparations, several penicilloyl-RSA, penicilloyl-HSA, and penicilloyl-PLL preparations. Color values per μg conjugate protein for RGG and the HGG conjugates were identical (difference less than 3 per cent). Color values per μg protein for the serum albumin conjugates were 40 to 48% of that for γ-globulin; those for PLL conjugates were 50 per cent of the color value for λ-globulin. In the zone of antibody excess, the weight of antibodies precipitated from serum was obtained by subtracting the weight of antigen added (multiplied by the ratio, $\frac{\text{color value antigen}}{\text{color value globulin}}$) from the total protein precipitated. Supernatants were negative for antigen in the antibody excess zone, and gave weak positive reactions at the zone of maximal antibody precipitation (equivalence). It was arbitrarily assumed that approximately 10 per cent of the added antigen was not precipitated at equivalence, and this additional correction was used in calculating the weights of antibodies precipitated at equivalence. If the amount of antigen remaining soluble at equivalence is actually 0 to 20 per cent, the maximum error introduced into the weights of antibodies precipitated at equivalence would be 1 per cent for antibodies precipitated by PLL conjugates, and 4 per cent for antibodies

precipitated by HGG conjugates. The maximum over-all experimental error in these analyses was judged to be ±5 to 7 per cent.

Quantitative Hapten Inhibition of Precipitation.—Methods developed by Landsteiner (19) and by Pauling *et al.* (20) were used. For experiments in Fig. 5, haptens at increasing concentrations dissolved in 1.00 ml B.S., pH 7.3 were mixed with 0.50 ml aliquots of the clarified rabbit anti-BPO globulin fraction, followed immediately by the addition of sufficient antigen dissolved in 0.50 ml B.S. to reach equivalence. For the experiments in Figs. 3 and 4, haptens dissolved in 0.50 ml B.S. were mixed with 1.00 ml globulin fraction followed by the addition of 500 μg antigen in 0.20 ml B.S. These mixtures as well as uninhibited antigen-globulin fraction mixtures and antigen and globulin fraction controls diluted in B.S. were set up in duplicate. Tubes were incubated, and precipitates were washed and analyzed by the Folin method as described above; mean deviation, ±3 per cent. Maximum precipitation was achieved under these conditions. The weights of protein precipitated from the uninhibited antigen-globulin fraction mixtures were: 240 μg for experiments in Fig. 5; and 80 μg for experiments in Figs. 3 and 4. Antigen and globulin fraction controls precipitated a total of 5 to 10 μg protein. Undecomplemented globulin fraction was used in these experiments as the curves obtained from inhibition of precipitation of the globulin fraction by BPO_{22}-HGG with BPO-EACA[1] were identical for decomplemented and undecomplemented globulin fraction. Repeat hapten inhibition curves were done for several haptens at different times and using different hapten preparations. Mean deviation in hapten concentration required for 50 per cent inhibition of precipitation was ±5 per cent.

RESULTS

Analysis of Conjugates.—Table I shows the comparisons between the numbers of penicilloyl groups introduced into various conjugates by reaction with penicillenic acids, and the numbers of lysine ϵ-NH_2 groups blocked. The two figures are in good agreement, demonstrating that penicilloyl groups are bound covalently to carrier proteins predominantly as penicilloyl-lysine groups. Ultraviolet absorption spectra of the penicilloyl-protein and penicilloyl-PLL[1] conjugates exhibited absorption peaks at 285 mμ as described and discussed previously (7). The penicilloyl conjugates were free of detectible penicillenic disulfide groups by ultraviolet spectrophotometric analysis (7), except for the DMPPO-protein conjugates. After several treatments of the DMPPO-protein conjugates with cysteine and with mercaptoethylamine, they still exhibited an absorption shoulder at 340 mμ with optical densities corresponding to less than one penicillenic disulfide group per mole conjugate. The phenylacetyl-protein conjugates (prepared by reaction of phenylacetyl chloride with proteins) contained 14–26 fewer ϵ-NH_2 groups per mole than did the native proteins (Table I). Thus the conjugates contained at least that number of phenylacetylamine groups bound covalently to lysine side chains.

Absence of Antibodies Complementary to the Phenylacetylamine End of the BPO Molecule.[7]—In the following experiments the globulin fraction of a pooled

[7] The phenylacetylamine group could act as an antigenic determinant. Five guinea pigs sensitized with PAC_{15}-guinea pig albumin exhibited strong Arthus and delayed skin reactivity to the immunizing conjugate and negative reactions to native and acetylated guinea pig albumin (unpublished Experiments).

rabbit anti-BPO serum (prepared by immunization with benzylpenicillin)[8] was analyzed for the presence of anti-phenylacetylamine antibodies, and anti-thiazolidine carboxylic acid antibodies. Fig. 2 shows that no anti-phenylacetylamine antibodies could be detected in the globulin fraction by quantitative precipitation with PAC_{22}-HGG, PAC_{26}-RGG and PAC_{14}-RSA; whereas the

TABLE I

Analysis of Penicilloyl and Phenylacetyl Conjugates of Various Proteins

Conjugate*	Preparation	Penicilloyl groups per mole conjugate (average)‡	Lysine-NH_2 groups blocked per mole conjugate (average)
BPO-HGG	A	22	20
DMPPO-HGG	A	22	25
AMMPO-HGG	A	42	—‖
BPO-RSA	A	27	—
	B	18	—
	C	4.4	3.6
BPO-HSA	A	5.0	5.2
	B	13	10
DMPPO-RSA	A	27	27
	B	13	13
	C	22	23
	D	18	18
DMPPO-HSA	A	19	15
	C	15	15
BPO-PLL	A	5.6	—
DMPPO-PLL	A	5.6	—
PAC-HGG	A	0	22
PAC-RGG	A	0	26
PAC-RSA	A	0	14

* See footnote 1 for abbreviations.

‡ Penicilloyl analysis by penamaldate method; protein by micro-Kjeldahl corrected for nitrogen contribution of penicilloyl groups. Molecular weights: HGG, 160,000; RSA, 69,000; HSA, 69,000; PLL 20 units average, 2,600; RGG, 160,000 (See Experimental).

§ Lysine ϵ-NH_2 by formal titration, protein analyzed as above. ϵ-NH_2 groups per mole found for native proteins were: HGG, 89; RSA, 58; HSA, 56; RGG, 88.

‖ (—) analyses not done.

globulin fraction contained 265 μg per ml antibody precipitable by BPO_{22}-HGG. No anti-phenylacetylamine antibodies could be detected by PCA in guinea pigs (21) using the globulin fraction in 1 to 20 dilution and 1 mg of the PAC-protein conjugates as antigens, whereas with 250 μg of BPO_{22}-HGG as

[8] Immunization of rabbits with benzylpenicillin results in the formation of anti-BPO antibodies. It is believed that benzylpenicillin rearranges to benzylpenicillenic acid *in vivo* which reacts with tissue proteins to form antigenic BPO-protein conjugates (7).

antigen, the globulin fraction gave positive PCA reactions to a dilution of 1 to 2500.

Absence of Antibodies Complementary to the Thiazolidine–Carboxylic Acid End of the BPO Molecule.—Anti-BPO antibodies which encompass only the thiazolidine carboxylic acid portion of the BPO molecule should be precipitated equally well by conjugates containing AMMPO or DMPPO groups provided

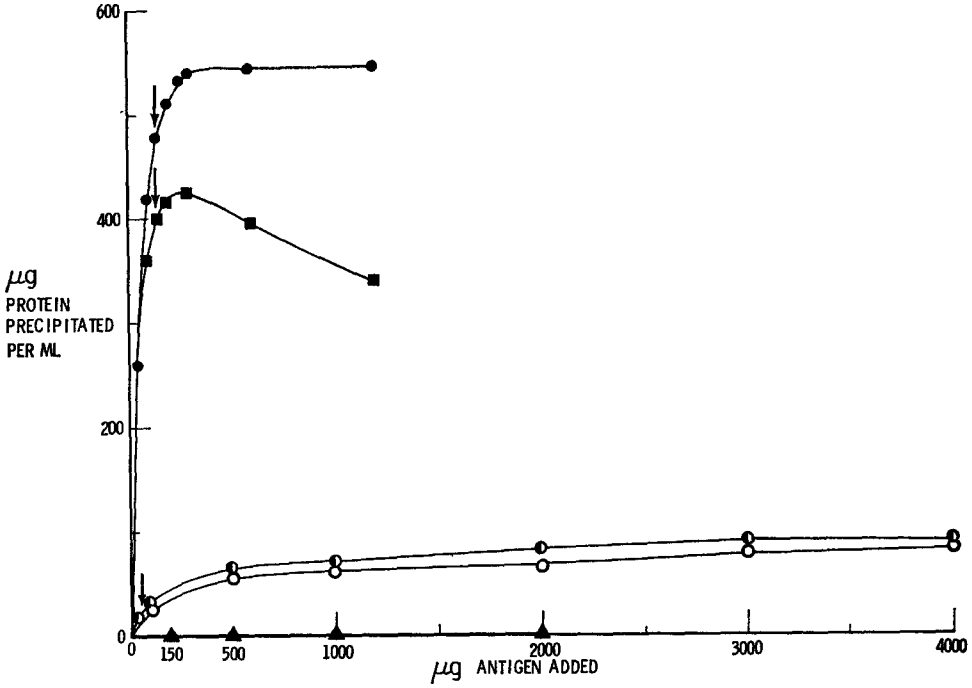


FIG. 2. Precipitation of rabbit anti-benzylpenicilloyl pooled serum (globulin fraction) by: ● BPO_{22}-HGG; ■, BPO_{22}-HGG (decomplemented globulin fraction); ○, $DMPPO_{22}$-HGG; ◐, $AMMPO_{42}$-HGG; ▲ PAC_{14}-RSA, PAC_{26}-RGG, PAC_{22}-HGG. (↓) indicates point of maximal antibody precipitation.

that there is no steric interference from the N-7 side chain. Fig. 1 shows that these penicilloyl groups are identical to the BPO group with regard to the structure of the thiazolidine carboxylic acid portion of the molecule, and differ only in the structure of the side chain on the N-7 nitrogen atom. Also, Fisher-Hirschfelder-Taylor scale models of these molecules (Fig. 1) show that the thiazolidine carboxylic acid ends of these penicilloyl molecules may be presented to a hypothetical anti-thiazolidine binding site without steric interference from the N-7 side chain.

Figure 2 shows the results of quantitative precipitation analysis of the anti-BPO globulin fraction using BPO_{22}-HGG, $DMPPO_{22}$-HGG and $AMMPO_{42}$-

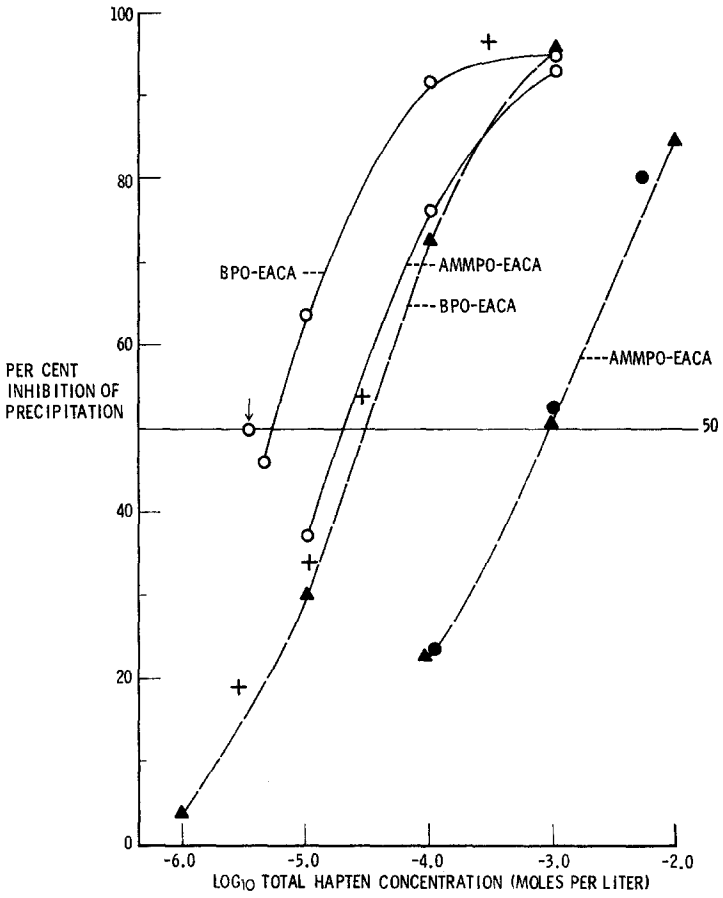


FIG. 3. Hapten inhibition of the precipitation of pooled rabbit anti-BPO globulin fraction by $AMMPO_{42}$-HGG, shown by solid lines. ↓ ○ indicates the corrected BPO-EACA concentration required for 50 per cent inhibition (see footnote 11). For comparison is shown hapten inhibition of precipitation of the anti-BPO globulin fraction by BPO_{22}-HGG (broken lines; data from Fig. 5). Also shown is hapten inhibition of the precipitation of anti-BPO globulin fraction (which had been absorbed with $DMPPO_{22}$-HGG) by $AMMPO_{42}$-HGG. Haptens are: +, BPO-EACA; ●, AMMPO-EACA.

HGG as antigens. The $DMPPO_{22}$-HGG and $AMMPO_{42}$-HGG antigens precipitated a maximum of 9 per cent[9] of the weight of antibodies precipitated by

[9] The maximum amount of antibody protein precipitable from the globulin fraction by $DMPPO_{22}$-HGG and $AMMPO_{42}$-HGG was 25 μg. per ml., part of which is probably complement protein. In comparison BPO_{22}-HGG precipitated 345 μg. per ml. protein from the undecomplemented globulin fraction and 265 μg. protein per ml. from the decomplemented globulin fraction. The difference which is presumably complement protein would be 80 μg. per ml. providing the Folin color value for complement protein is equal to that for γ-globulin.

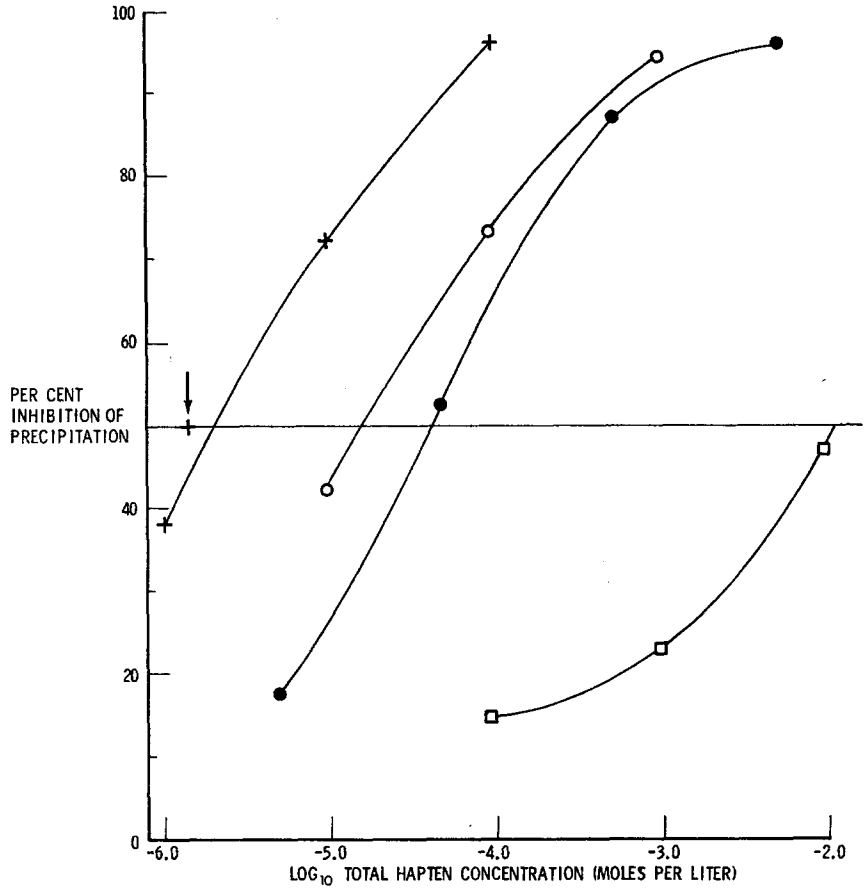


FIG. 4. Hapten inhibition of the precipitation of pooled rabbit anti-BPO globulin fraction by $DMPPO_{22}$-HGG with BPO-*n*-propylamine (+); and with DMPPO-*n*-propylamine (○). $(\overset{\downarrow}{+})$ indicates the corrected BPO-*n*-propylamine concentration required for 50 per cent inhibition (see footnote 11). For comparison is shown hapten inhibition of precipitation of the anti-BPO globulin fraction by BPO_{22}-HGG with BPO-*n*-propylamine (●), and with DMPPO-*n*-propylamine (□) (data from Fig. 5).

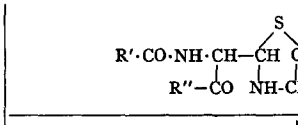
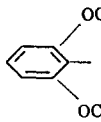
BPO_{22}-HGG. PCA analysis in guinea pigs (21) showed the globulin fraction to give positive reactions to a dilution of 1 to 2500 with BPO_{22}-HGG as antigen and to a dilution of 1 to 100 with $DMPPO_{22}$-HGG as antigen. The maximum amount of antibodies in the globulin fraction which could be specific for only the thiazolidine carboxylic acid portion of the BPO molecule is 9 per cent of the antibodies detectible by BPO_{22}-HGG. The other 91 per cent required for detection a conjugate containing the entire BPO structure. If the anti-BPO antibodies precipitable by $DMPPO_{22}$-HGG and $AMMPO_{42}$-HGG are indeed complementary to only the thiazolidine carboxylic acid end of the penicilloyl

molecule, the precipitation of these antibodies should be inhibited equally well by univalent haptens which contain the BPO, AMMPO or DMPPO groups.

Fig. 3 shows the results of quantitative hapten inhibition experiments which compare the abilities of BPO-EACA and AMMPO-EACA to specifically inhibit the precipitation of the anti-BPO globulin fraction by $AMMPO_{42}$-HGG, and Fig. 4 shows the comparative abilities of BPO-*n*-propylamine and

TABLE II

Decrease in Standard Free Energy of Binding, ($-\Delta F°$), of Penicilloyl Haptens to the Anti-BPO Globulin Fraction

Hapten			$\Delta(-\Delta F)$ (BPO-EACA – Other Hapten)
	R′	R″	
			cal per mole
BPO-EACA	$C_6H_5CH_2-$	$^{\ominus}OOC-(CH_2)_5-NH-$	—
BPO-*n*-octylamine	"	$CH_3(CH_2)_7-NH-$	0
BPO-*n*-amylamine	"	$CH_3(CH_2)_4-NH-$	170
BPO-*n*-propylamine	"	$CH_3(CH_2)_2-NH-$	170
BPO-benzylamine	"	$C_6H_5-CH_2-NH-$	170
BPO-amine	"	NH_2-	460
Benzylpenicilloate	"	$^{\ominus}O-$	1450
AMMPO-EACA	$CH_2=CH-CH_2-S-CH_2-$	$^{\ominus}OOC-(CH_2)_5-NH-$	1900
DMPPO-*n*-propylamine		$CH_3(CH_2)_2-NH-$	3300
Phenylacetylglycine	$C_6H_5CH_2CONHCH_2COO^{\ominus}$	—	3500

$$\Delta(\Delta F°) = -2.303\ RT \log_{10} \frac{C_{50}\ \text{BPO-EACA}}{C_{50}\ \text{other hapten}}$$ (Kabat, reference 1).

* Equilibrium diastereoisomeric mixture of penicilloyl haptens.

‡ Assumed $\Delta F°$ for BPO-EACA, −7500 cal/mole (23–25).

DMPPO-*n*-propylamine to specifically inhibit the precipitation of the globulin fraction by $DMPPO_{22}$-HGG[10]. Chemical structures of the haptens are shown

[10] The penicilloyl haptens were used as their sterically equilibrated diasteriosomeric mixtures (14) since rabbit anti-BPO antibodies prepared by immunization with benzylpenicillin are specific for the diasteriosomeric BPO groups rather than for the α-diastereoisomer (7). The molar specific rotations of the sterically equilibrated diastereoisomeric mixtures of the BPO-amines and of AMMPO-EACA were essentially identical (14). Thus it is probable that the ratio of diasterioisomers present at equilibrium is the same for these penicilloyl amines. Also it appears likely that the ratio is the same for BPO-lysine groups in BPO conjugates prepared by reaction of proteins with D-benzylpenicillenic acid. These ratios appear to differ from the ratios of diastereoisomers present in the sterically equilibrated diastereoisomeric mixtures of D-α-benzylpenicilloate (see Experimental) and DMPPO-*n*-propylamine (14).

in Table II. In both systems, the BPO hapten was the more effective inhibitor. Precipitation of globulin fraction by $AMMPO_{42}$-HGG was inhibited 4.9 times as effectively by BPO-EACA as by AMMPO-EACA; hapten concentrations required for 50 per cent inhibition were 4.1×10^{-6} moles/liter BPO-EACA and 2.0×10^{-5} moles/liter AMMPO-EACA.[11-13] Precipitation of the globulin fraction by $DMPPO_{22}$-HGG was inhibited 11.7 times as effectively by BPO-*n*-propylamine than by DMPPO-*n*-propylamine; hapten concentrations required for 50 per cent inhibition were 1.4×10^{-6} moles/liter BPO-*n*-propylamine and 1.6×10^{-5} moles/liter DMPPO-*n*-propylamine.[11-13]

Although the anti-BPO antibodies precipitated by $AMMPO_{42}$-HGG and $DMPPO_{22}$-HGG could distinguish the BPO hapten from the AMMPO and DMPPO haptens, these "cross-reacting" anti-BPO antibodies distinguished less well between BPO and AMMPO or DMPPO haptens than did the anti-BPO antibodies requiring BPO_{22}-HGG for precipitation. For example, Fig. 3 shows that the precipitation of the anti-BPO globulin fraction by $AMMPO_{42}$-HGG required for 50 per cent inhibition 4.9 times as high a molar concentration of AMMPO-EACA, as of BPO-EACA, whereas precipitation of the globulin fraction by BPO_{22}-HGG required for 50 per cent inhibition 30 times as high as molar concentration of AMMPO-EACA as of BPO-EACA.[13] Further, when the globulin fraction was first absorbed with $DMPPO_{22}$-HGG, precipitation of the

[11] The total hapten concentrations required for 50 per cent inhibition of precipitation of the globulin fraction by $AMMPO_{42}$-HGG were corrected for specific binding of these haptens by the anti-BPO antibodies which are not precipitated by $AMMPO_{42}$-HGG. This correction was calculated from the mass action Law assuming average association constants for antibody hapten binding of 10^6 for BPO-EACA and 10^4 or 10^5 for AMMPO-EACA. The total antibody-combining site concentration in the inhibition mixtures was 1.7×10^{-6} moles/liter. For BPO-EACA, the observed total hapten concentration required for 50 per cent inhibition was 5.4×10^{-6} moles/liter, and the correction for non-precipitable antibody-bound hapten amounted to 1.3×10^{-6} moles/liter. For AMMPO-EACA, the observed total hapten concentration required for 50 per cent inhibition was 2.0×10^{-5} moles/liter, and the correction for non-precipitable antibody-bound hapten was less than 1×10^{-6} moles/liter, and is thus negligibly small. Similar corrections were made for total hapten concentrations required for 50 per cent inhibition of precipitation of the globulin fraction by $DMPPO_{22}$-HGG. For BPO-*n*-propylamine the observed total hapten concentration required for 50 per cent inhibition was 2.3×10^{-6} moles/liter and the correction amounted to 0.9×10^{-6} moles/liter. The correction for DMPPO-*n*-propylamine was negligibly small.

[12] Inhibition was specific as, in the concentrations used, these haptens did not inhibit precipitation of rabbit anti-ovalbumin and rabbit anti-bovine γ-globulin antibodies by their respective antigens.

[13] Inhibition was carried out on the AMMPO-HGG- and the DMPPO-HGG-precipitating systems in far antigen excess. This was done to increase the amount of protein precipitated (there is no inhibition of precipitation in far antigen excess, Fig. 2) and to increase also the concentration of haptens required for 50 per cent inhibition. For the BPO-HGG precipitating system, inhibition was carried out at equivalence. The ratio of the concentrations of haptens (*i.e.* AMMPO-EACA *versus* BPO-EACA) required for 50 per cent inhibition is the same at equivalence as it is in far antigen excess (20).

remaining anti-BPO antibodies by BPO_{22}-HGG required for 50 per cent inhibition 38 times as high a molar concentration of AMMPO-EACA as of BPO-EACA (Fig. 3). Similarly, Fig. 4 shows that precipitation of the anti-BPO

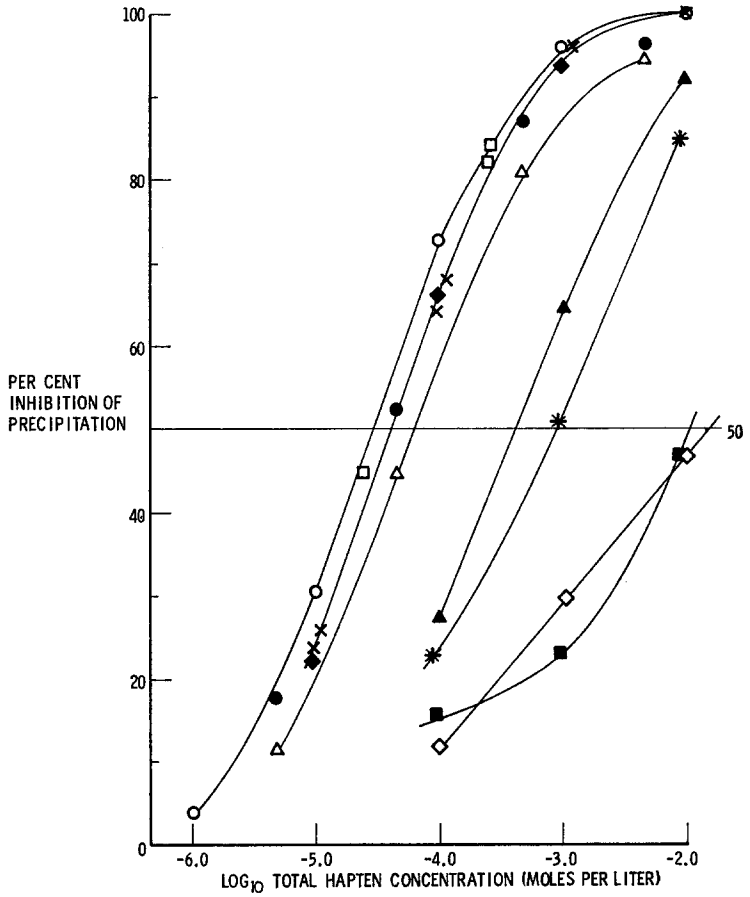


FIG. 5. Hapten inhibition of precipitation of the pooled rabbit anti-BPO globulin fraction by BPO_{22}-HGG. Penicilloyl haptens are equilibrium diastereoisomeric mixtures (see Table II for structural formulas). ○, BPO-EACA; □, BPO-*n*-octylamine; x, BPO-*n*-amylamine; ●, BPO-*n*-propylamine; ◆, BPO-benzylamine; △, BPO-amine; ▲ benzylpenicilloate; *, AMMPO-EACA; ■, DMPPO-*n*-propylamine; ◇ phenylacetylglycine.

globulin fraction by $DMPPO_{22}$-HGG required for 50 per cent inhibition 12 times as high a concentration of DMPPO-*n*-propylamine as of BPO-*n*-propylamine whereas precipitation of the globulin fraction by BPO_{22}-HGG required 260 times as high a concentration of DMPPO-*n*-propylamine as of BPO-*n*-propylamine.[13]

Quantitative Hapten Inhibition of Precipitation of the Anti-BPO Globulin Fraction by BPO_{22}-HGG. In previous experiments with pooled rabbit anti-BPO whole sera (prepared by immunization of rabbits with benzylpenicillin), hapten inhibition experiments indicated that the anti-BPO combining sites were complementary also for the lysine side of carrier proteins (7). In the present experiments, an albumin-free globulin fraction of a pooled rabbit anti-BPO serum was studied to avoid reversible binding of haptens by serum albumin (22). A group of BPO amine[10] univalent haptens which differ in the lengths of the amide side chains (see Table II for chemical structures) as well as free benzylpenicilloic acid, AMMPO-EACA, DMPPO-*n*-propylamine, and phenylacetylglycine, were compared with regard to their abilities to specifically inhibit precipitation of the rabbit anti-BPO pooled serum globulin fraction by BPO_{22}-HGG.[12] Results are plotted, per cent inhibition versus log total hapten concentration as suggested by Pauling *et al.* (20) in order to facilitate expression of the data in energetic terms. The results in Fig. 5 show a family of parallel sigmoid inhibition curves indicating that the anti-BPO-globulin fraction shows the same degree of heterogeneity in antibody-hapten binding energies toward each of these haptens (20). Under these conditions, the difference in the log molar concentrations of two haptens required to achieve 50 per cent inhibition is proportional to the difference in average standard free energy of hapten binding to antibody ($-\Delta F^\circ$) between these two haptens (20). Table II lists $\Delta(-\Delta F^\circ)$ between BPO-EACA and the other haptens calculated from the formula given by Kabat (1). The most effective inhibitor was BPO-EACA; the effectiveness of the other BPO-amines was intermediate between the effectiveness of BPO-EACA and BPO-amine. The sharp drop in $-\Delta F^\circ$ between BPO-amine and benzylpenicilloate may be due to repulsive effects of the carboxylate ion and the change in electronic distribution induced in the BPO molecule by this ion. This decreased binding affinity may be due also to the difference between the ratio of diastereoisomers in the sterically equilibrated benzylpenicilloate solution, and this ratio in solutions of sterically equilibrated BPO-amines.[10] Similarly, this steric factor may be responsible in part for the relatively poor binding of DMPPO-*n*-propylamine. The equal inhibitory effectiveness of the water-soluble BPO-EACA and the poorly soluble BPO-*n*-octylamine indicates that these inhibition results reflect specific antibody-binding differences rather than reflecting non-immunological properties of these haptens (*e.g.*, solubility).

Precipitation of Rabbit Anti-(BPO-RSA) Sera with Penicilloyl Conjugates of RSA and Heterologous Carriers.—Sera from individual rabbits were used and decomplemented to avoid coprecipitation of complement protein (17). Neither native or acetylated RSA precipitated antibodies from these sera, and for sera 6 and 34, precipitation at equivalence by penicilloyl conjugates was inhibited completely by 1.0×10^{-2} M BPO-EACA. For mixtures of serum 5 with BPO-

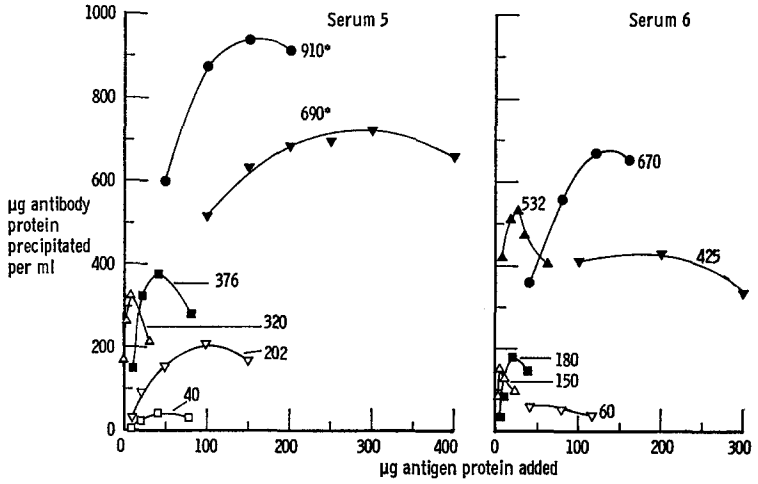


FIG. 6. Precipitation of decomplemented rabbit anti-(BPO_{27}-RSA) sera 5 and 6 with : ●, BPO_{27}-RSA; ▼, BPO_{22}-HGG; ▲, $BPO_{5.6}$-PLL; ■, $DMPPO_{27}$-RSA; △, $DMPPO_{5.6}$-PLL; ▽, $DMPPO_{22}$-HGG; □, $DMPPO_{15}$-HSA. The numbers refer to the maximum weights (μg per ml) of antibodies precipitated. The figures with asterisks have been corrected for "non-BPO antibody." (see text).

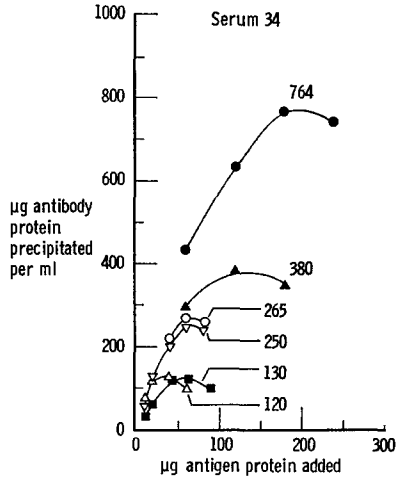


FIG. 7. Precipitation of decomplemented rabbit anti ($BPO_{4.4}$-RSA) serum 34 with: ●, BPO_{27}-RSA: ▲, BPO_{13}-HSA; ○, $DMPPO_{18}$-RSA; ▽, $DMPPO_{13}$-RSA; △, $DMPPO_{22}$-RSA; ■, $DMPPO_{15}$-HSA.

EACA, amounts of BPO_{22}-HGG and BPO_{27}-RSA sufficient to reach equivalence precipitated less than 30 μg antibody protein per ml, or 3 to 4 per cent of the weight of antibodies precipitated by these antigens at equivalence. This small correction for "non-BPO" antibody was made. Thus, antibodies precipitated

from the three sera were judged to be specific for the BPO group. The results of quantitative precipitation analyses are shown in Figs. 6, 7. Penicilloyl conjugates of the immunizing protein carrier (RSA) precipitated more antibodies from serums 5 and 6 than did penicilloyl conjugates of heterologous carriers (HGG, HSA, and PLL). This "carrier effect" was more pronounced for the anti-BPO antibodies which were precipitated by DMPPO conjugates (termed cross-reacting antibodies) than it was for the "non-cross-reacting" antibodies which required BPO conjugates for precipitation. For example, for serum 5, $DMPPO_{27}$-RSA precipitated 1.9 times the weight of antibodies precipitated by $DMPPO_{22}$-HGG, whereas BPO_{27}-RSA precipitated only 1.1 times the weight of non-cross-reacting anti-BPO antibodies[14] precipitated by BPO_{22}-HGG (Fig. 6). For serum 6, $DMPPO_{27}$-RSA precipitated 3.0 times the weight of anti-BPO antibodies precipitated by $DMPPO_{22}$-HGG, whereas BPO_{27}-RSA precipitated only 1.4 times the weight of non-cross-reacting antibodies precipitated by BPO_{22}-HGG. The "carrier effect" was least for the PLL conjugates, and most marked for the HSA conjugates (Figs. 6, 7). Fig. 7 shows also a comparison of the abilities of three different DMPPO-RSA preparations to precipitate anti-BPO antibodies from serum 34 (rabbit anti-BPO_4-RSA). Two different DMPPO-RSA conjugates which were prepared in the same way (1 molar equivalent dimethoxyphenylpenicillenic acid per mole lysine ϵ-NH_2, pH 7.5–8.0, 6 per cent ethanol) precipitated equal weights of antibodies from the serum, *i.e.* $DMPPO_{18}$-RSA (prep. D), 265 μg per ml $\pm$ 5 per cent; $DMPPO_{13}$-RSA (prep. B) 250 μg per ml $\pm$ 5 per cent. A third preparation, $DMPPO_{22}$-RSA (prep. C), prepared under more severe conditions, (2.5 molar equivalents of the penicillenic acid per mole lysine ϵ-NH_2, pH 8.0–9.0, 15 per cent ethanol) precipitated only 130 μg per ml $\pm$ 5 per cent of antibodies from the serum. All 3 conjugates were prepared from the same penicillenic acid and RSA preparations, but at different times.

DISCUSSION

The foregoing experimental results demonstrate that at least a large portion of rabbit antibodies prepared against the benzylpenicilloyl (BPO) bifunctional haptenic group are specifically adapted to a large antigenic unit consisting of the entire BPO group, the lysine side chain through which BPO groups are bound predominantly to proteins, and structural areas of the immunizing carrier protein (probably adjoining the point of attachment of the BPO group). No antibodies which are specifically adapted to only the phenylacetylamine or

[14] The weight of non-cross-reacting anti-BPO antibodies was obtained by subtracting the weight of antibodies precipitated by a DMMPO conjugate from the weight of antibodies precipitated by its homologous BPO conjugate. After absorption of a serum with BPO_{27}-RSA or with BPO_{22}-HGG, no additional antibodies could be precipitated by $DMPPO_{27}$-RSA or $DMPPO_{27}$-HGG.

the thiazolidine carboxylic acid portions of the bifunctional BPO molecule could be detected.

Of interest is the observation that phenylacetylglycine could bind specifically to a pooled rabbit anti-BPO serum globulin fraction (Fig. 5) whereas several phenylacetylamine-protein conjugates could not detectibly react with the anti-BPO globulin fraction by specific precipitation (Fig. 2) or by PCA reaction. These findings are consistent with the view that the antibody-combining site is an intramolecular cavity (23) rather than a specific patch on the surface of the antibody molecule. Thus, whereas the low molecular weight phenylacetylglycine molecule can pass unrestricted into the large combining site cavity to reach its specific combining surface, such a passage may be impossible for phenylacetylamine residues bound closely to a high molecular weight protein.

Rabbit anti-BPO antiserums could be fractionated into two distinct fractions of anti-BPO antibodies on the basis of their abilities to cross-react with dimethoxyphenylpenicilloyl (DMPPO) and allylmercaptomethylpenicilloyl (AMMPO) conjugates. For the pooled rabbit anti-BPO serum globulin fraction, a maximum of 9 per cent of the weight of antibodies precipitated by BPO_{22}-HGG could be precipitated by $DMPPO_{22}$-HGG or by $AMMPO_{42}$-HGG (Fig. 2). Based on quantitative hapten inhibition data, anti-BPO antibodies in the cross-reacting fraction (9 per cent) as well as in the non-cross-reacting fraction, (91 per cent) appear to be specifically adapted to the entire BPO molecule rather than to the thiazolidine carboxylic acid portion of the BPO molecule alone. Specific precipitation of both fractions was inhibited more effectively by BPO haptens than by homologous AMMPO or DMPPO univalent haptens (Figs. 3, 4). However, the non-cross-reacting anti-BPO antibodies (which require BPO-conjugates for precipitation) could distinguish between the BPO and AMMPO or DMPPO haptens better than could the cross-reacting anti-BPO antibodies. For example, a 38-fold greater molar concentration of AMMPO-EACA than BPO-EACA was required for 50 per cent inhibition of precipitation of the non-cross-reacting anti-BPO antibodies, whereas only a 4.9-fold greater molar concentration of AMMPO-EACA than BPO-EACA was required for 50 per cent inhibition of precipitation of the cross-reacting anti-BPO antibodies. Thus, the cross-reacting anti-BPO antibodies appear to be less closely fitted to the haptenic groups (*i.e.*, less avidly bound) than are the non-cross-reacting anti-BPO antibodies. The possibility cannot be excluded, however, that the cross-reacting fraction of anti-BPO antibodies contains some antibody molecules with specificity only for the thiazolidine carboxylic acid portion of the BPO group. Regardless of this possibility, it is clear that at least the bulk of anti-BPO antibodies are specifically adapted to the entire BPO haptenic group.

Anti-BPO antibodies are specifically adapted also to the 6 carbon lysine

side chain through which penicilloyl groups are bound predominantly to proteins. Thus, BPO-EACA (with a 6 carbon amide side chain) was a more effective specific inhibitor of precipitation of anti-BPO antibodies than were BPO-amines with smaller amide side chains (Fig. 5). The contribution made by the 6 carbon chain to specific antibody hapten binding was 460 cal per mole, [$\Delta(-\Delta F^\circ)$ between BPO-EACA and BPO-amine, see Table II.] Assuming an average $-\Delta F^\circ$ for antibody binding of BPO-EACA to be 7500 cal per mole,[15] the binding contribution for the 6 carbon side chain is small, roughly 6 per cent. In comparison for the dinitrophenyl (DNP) system, the contribution made to specific binding by the lysine side chain is considerably larger. Using hapten dissolution of precipitates, Farah *et al.* (26) found ϵ-DNP-lysine to be 30 times as effective as was DNP-glycine, corresponding to a $\Delta(-\Delta F^\circ)$ of 1900 cal per mole. Also, Velick *et al.* (27) using fluorescent quenching techniques found, for a purified rabbit anti-DNP antibody preparation, association constants of 4.2×10^8 and 1.4×10^6 for the binding of ϵ-DNP-lysine and DNP-acetate respectively. These figures correspond to a $\Delta(-\Delta F^\circ)$ of 3200 cal per mole, where $-\Delta F^\circ$ for the binding of ϵ-DNP-lysine was 11,300 cal. per mole. Although more data along these lines are needed, the difference in the contribution made by the lysine side chain to antibody hapten binding for the DNP and BPO systems may be ascribed provisionally to the difference in size of these haptens.

Anti-BPO antibody-combining sites show specificity also for structures of the immunizing carrier protein. Thus for three antisera from individual rabbits immunized with BPO-RSA, penicilloyl conjugates of the immunizing carrier (RSA) precipitated more antibodies than did conjugates of three heterologous carriers (PLL, HGG, and HSA) (Figs. 6, 7). The different precipitation abilities found for conjugates of three different heterologous carriers (Figs. 6, 7) suggest that these abilities may be defined also by factors other than specific structural adaptation between the combining site and the carrier. Thus non-specific protein-protein interactions between the antibody and antigen molecules, as well as repulsive forces between the antibody-combining site and the more or less poorly fitted heterologous carrier surface, would contribute also to the total binding between antigen and antibody. Further, solubility factors may also determine, in part, the ability of a conjugate to precipitate antibodies.

The contribution made by the carrier to precipitation of anti-BPO antibodies was considerably greater for antibodies precipitated by the DMPPO conjugates (relatively poorly fitted to hapten) than it was for the anti-BPO antibodies which require BPO conjugates for precipitation (relatively closely fitted to hapten) (Figs. 6, 7). These results suggest that as the binding energy contributed by antibody-hapten interaction increases, the binding contribution made by the carrier becomes less important for precipitation. Accordingly,

[15] Values of this order have been found for other hapten-antibody interactions (23–25).

sufficiently avid anti-hapten antibodies should be precipitated equally well by conjugates of the immunizing and heterologous carriers (providing they are sufficiently highly conjugated). Thus the different degrees of "carrier specificity" demonstrated by the three sera (Figs. 6, 7) may be explained equally well by heterogeneity of anti-BPO antibodies with regard to avidity of binding to the hapten, as it can by heterogeneity of anti-BPO antibodies with regard to the dimensions of their combining sites (*Cf.* reference 1).

Of interest is the data in Fig. 7 showing that $DMPPO_{22}$-RSA prepared under "denaturing" conditions precipitated considerably fewer antibodies from serum 34 (rabbit anti-BPO_4-RSA) than did $DMPPO_{13}$-RSA and $DMPPO_{18}$-RSA which were prepared under comparatively "mild" conditions as was the immunizing antigen, BPO_4-RSA. Although preliminary, these results suggest that the method of preparation of a hapten conjugate may influence its ability to precipitate anti-hapten antibodies, especially antibodies of low binding affinity for hapten. The results suggest also that anti-BPO antibody-combining sites may be adapted to secondary and tertiary structural configurations of the immunizing carrier protein. The finding that a mixture of DNP-lysyl peptides (prepared by pepsin digestion at pH 1–2 of the immunizing protein conjugate, DNP-BGG), were not more effective than was ϵ-DNP-lysine in dissolving anti-DNP precipitates (26) might be due to the absence in the DNP-peptides of the specific secondary and tertiary structural configurations against which the antibody is adapted.

The findings presented above are in agreement with earlier works with smaller-sized haptenic groups showing carrier specificity for anti-hapten antibodies (3–5), and are consistent also with more recent demonstrations that human anti-dextran antibodies (1) and rabbit anti-silk fibroin antibodies (2) are adapted to relatively large antigenic units. The present findings differ from those of Landsteiner and van der Scheer (6) who concluded that the combining sites of rabbit antibodies produced against the SA and GIL bifunctional haptens (Fig. 1) were not sufficiently large to encompass the entire haptenic groups. Although these different results may reflect the differences between the chemical structures of the haptens used in the two studies, more work is necessary to clarify this apparent inconsistency.

Although it is clear that anti-BPO antibodies demonstrate specificity also for the protein carrier, the size of the complementary area shared by antibody and carrier protein is unknown. Accordingly the precise dimensions of the anti-BPO antibody-combining sites could not be determined. Nevertheless, it is clear that at least a large part of the anti-BPO antibodies are complementary to an antigenic unit which is larger than the BPO group (one steric form of which is 17Å in length, Fig. 1) (*Cf.* references 1, 2), and which comprises also the lysine side chain and other structural areas of the carrier protein.

SUMMARY

Rabbit antisera prepared against conjugates of the benzylpenicilloyl (BPO) bifunctional haptenic group were analyzed to determine whether the antibodies are adapted to only a portion of the large BPO molecule, or to the entire molecule, and whether specificity extends to the lysine side chain and adjoining structures of the immunizing carrier protein.

No antibodies adapted to the phenylacetylamine portion of the BPO group could be detected in a pooled rabbit anti-BPO serum globulin fraction by PCA and quantitative precipitin analysis using several phenylacetylamine-protein conjugates as antigens. No antibodies adapted only to the thiazolidine carboxylic acid portion of the BPO molecule were detected in the anti-BPO globulin fraction using quantitative precipitin and hapten inhibition methods. At least the bulk of the anti-BPO antibodies was found to be adapted to the entire BPO haptenic group.

By quantitative hapten inhibition of precipitation of the anti-BPO globulin fraction, the anti-BPO antibodies were found to show specificity for a 6 carbon amide side chain corresponding to the lysine side chain through which BPO groups are bound predominantly to protein. The contribution of this 6 carbon chain to antibody-hapten binding was small; $(-\Delta F^{\circ})$ was calculated to be 460 calories per mole (average).

Rabbit anti-BPO antibodies prepared against BPO-rabbit serum albumin conjugates showed specificity also toward structures of the immunizing carrier protein, and possibly toward secondary or tertiary structural configurations. Penicilloyl conjugates of rabbit serum albumin precipitated from 3 individual rabbit antisera more anti-BPO antibodies than did penicilloyl conjugates of heterologous carriers (poly-L-lysine, human serum albumin, and human γ-globulin).

Anti-BPO antibodies demonstrated heterogeneity with regard to closeness of fit to the haptenic group, or with regard to the dimensions of the combining sites, or both.

It was concluded that at least a large part of anti-BPO antibodies are specifically adapted to a large antigenic unit comprised of the entire BPO group, the lysine side chain, and structural configurations of the immunizing carrier protein.

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