STRUCTURE AND SPECIFICITY OF GUINEA PIG 7S ANTIBODIES

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In an earlier study (1), reproducible differences in structure were found among antibodies of different specificities from individual guinea pigs. The structural variations were revealed by dissociating the antibodies into their L and H polypeptide chains (2, 3). Starch gel electrophoresis of different dissociated antibodies revealed differences in the number and mobility of the multiple sharp bands corresponding to L chains. In striking contrast, the patterns of dissociated non-specific γ-globulins from the same animals showed diffuse zones in the L chain region.

The present experiments were designed to extend these observations and to answer several questions that arose in the course of the work. One set of questions concerns the effect of different procedures of immunization, the role of the carrier protein to which the haptens are conjugated, and the influence upon the electrophoretic patterns of fine differences in the immunologic specificity of the antibodies. The other group of questions bears upon the origin of the multiplicity of the L chain bands of antibodies directed against a single hapten and also upon the location of L chains in the antibody molecule.

Materials and Methods

Preparation of Hapten-Protein and Hapten-Polymer Conjugates.—Conjugates of bovine γ-globulin, bovine fibrinogen, and guinea pig albumin were prepared with dinitrofluorobenzene, picryl chloride, arsanilic acid, *p*-toluenesulfonyl chloride, benzoyl chloride, *p*-chlorobenzoyl chloride, and pentachlorobenzoyl chloride, using techniques described previously (1, 4). The following conventions are used to designate the conjugates: DNP-BGG, dinitrophenyl-bovine γ-globulin; Pic-GPA, picryl-guinea pig albumin; Tos-Ova, tosyl-ovalbumin; As-BSA, arsanilic-diazo-bovine serum albumin; B-GPA, benzoyl-guinea pig albumin; *p*-CB-GPA, *p*-chlorobenzoyl-guinea pig albumin; penta-CB-GPA, pentachlorobenzoyl-guinea pig albumin; Pips-GPA, *p*-iodophenylsulfonyl-guinea pig albumin.

Picryl-microgel (5) was kindly prepared by Dr. V. Shashoua of E. I. Dupont De Nemours and Company, Inc., Wilmington, Delaware, by reacting picryl chloride with polyacrylylhydrazine microgel.

Procedures of Immunization and Isolation of Purified Antibodies.—These were done exactly as described in a previous publication (1). Antibodies directed against DNP-polylysine were isolated by similar procedures from guinea pigs immunized with this polymer. The properties of the antigen and the immunologic response of the animals have been reported (6).

Reduction and Alkylation.—1 per cent solutions of the purified antibodies were reduced for

2 hours at room temperature in 8 M urea made 0.1 M in 2-mercaptoethanol. After reduction, iodoacetamide was added to a final concentration of 0.3 M and the solution was allowed to stand for 10 minutes at room temperature.

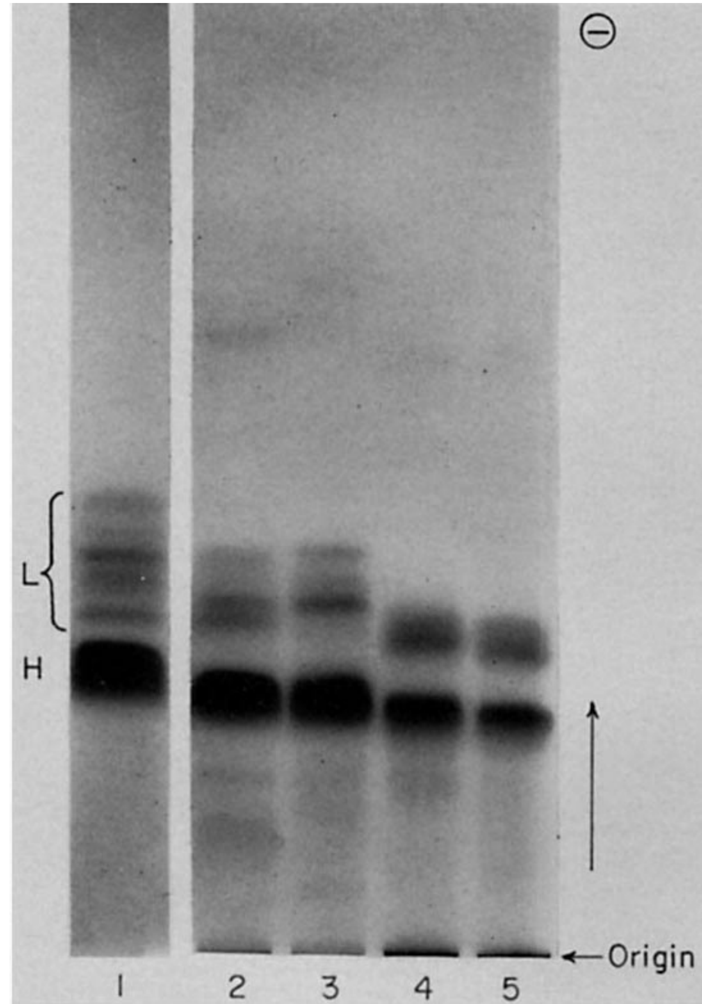

FIG. 1. Starch gel electrophoreses in urea-formate buffer of dissociated antibodies from individual guinea pigs.

1. Anti-Pic-GPA
2. Anti-Tos-Ova
3. Anti-Tos-GPA
4. Anti-As-GPA (animal 55-20)
5. Anti-As-GPA (animal 55-17)

All samples except No. 1 were electrophorized simultaneously. L,L chain bands; H,H chain bands.

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Starch Gel Electrophoresis in Urea-Formate Buffer.—The conditions for preparing the gel and performing the electrophoresis have been described (2). Following reduction and alkylation, the entire reaction mixture was placed in the origin of the starch-urea gel. It was found that alkylation was not required to obtain the characteristic patterns, since ionization of -SH groups at pH 3.0 is negligible. Nevertheless, slightly sharper bands resulted if alkylation was included in the procedure.

Hydrolysis with Papain.—Except as noted, the conditions were those described by Porter (7). The papain was 2 times recrystallized (Worthington Biochemical Corporation, Freehold, New Jersey).

Double Diffusion in Agar.—Undiluted antisera from individual guinea pigs and antigen solutions having concentrations of 0.5 per cent were used in conventional Ouchterlony plates. Purified antibody solutions were also used with similar results.

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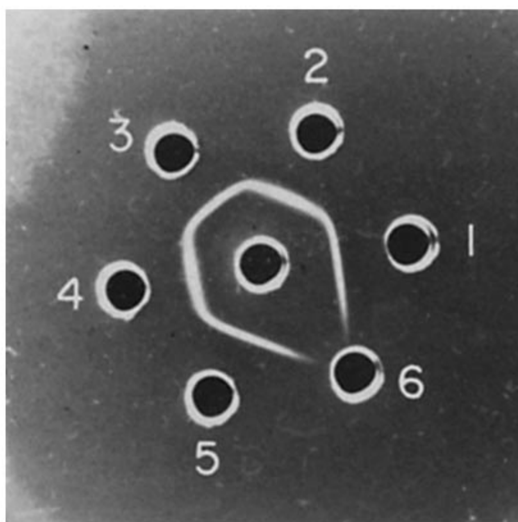

FIG. 2. Cross-reactivity of dinitrophenyl and picryl groups as tested by double diffusion in agar.

Central well, anti-DNP-GPA antiserum (animal 1–2)
1, 3, 5. DNP-GPA
2, 4. Pic-GPA
6. Pips-GPA

The concentration of all antigen solutions was 0.5 mg/ml.

RESULTS

I. Correlation between the Immunologic Specificity and the Starch Gel Electrophoretic Patterns of Dissociated Antihapten Antibodies.—With few exceptions dissociated antipicryl antibodies had starch gel electrophoretic patterns containing five bands corresponding to L chains (Fig. 1). Pools of antibodies from several animals also showed the same five bands after reduction and alkylation. Dissociated antipicryl or anti-DNP antibodies from single animals did differ,

however, in the intensity of staining of the different L chain bands. In general, no difference was found between the patterns of anti-DNP and antipicryl antibodies. These two systems cross-react extensively (Fig. 2).

The patterns of dissociated antiarsanilate, antitosyl, and antipicryl antibodies showed different numbers and distributions of bands (Fig. 1). Within a given specificity, the patterns were remarkably similar. As shown in Fig. 3,

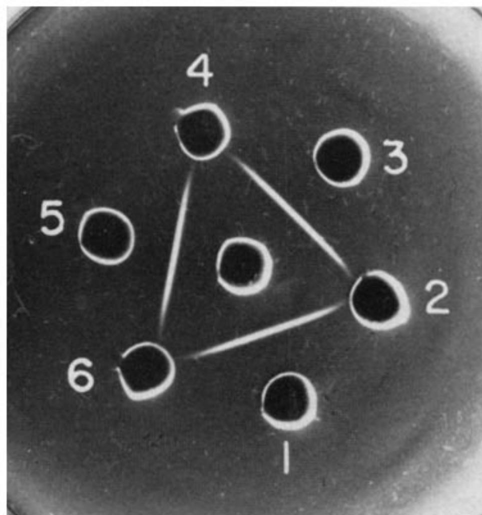

FIG. 3. Specificity of guinea pig anti-DNP antibodies.
Central well- anti–DNP-GPA antiserum (animal 1-2)
1, 3, 5. DNP-GPA
2. Tos-GPA
4. Pips-GPA
6. As-GPA
The concentration of all antigen solutions was 0.5 mg/ml.

anti-DNP antibodies did not precipitate with Tos-GPA and As-GPA when tested by double diffusion in agar.

All of the experiments described above were done using antibodies from animals immunized with antigen in complete Freund's adjuvant. Anti-DNP antibodies from animals immunized without adjuvants (8) also gave the characteristic patterns for this specificity. The dissociated γ-globulin of guinea pigs injected with complete adjuvant emulsified with 0.15 N NaCl showed diffuse L chain zones rather than bands.

Anti-DNP antibodies isolated from animals immunized with DNP-BGG had the same patterns as antibodies isolated from animals immunized with DNP-GPA. Dissociated antitosyl antibodies from animals immunized with

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Tos-Ova or Tos-GPA gave similar patterns (Fig. 1). Thus, variation of the carrier protein had little effect on the starch gel electrophoretic patterns of dissociated antihapten antibodies.

When DNP-polylysine was the immunizing antigen, the dissociated antibodies showed several kinds of patterns. One resembled that of antibodies from animals immunized with DNP-protein conjugates (Fig. 4, origin 2). The

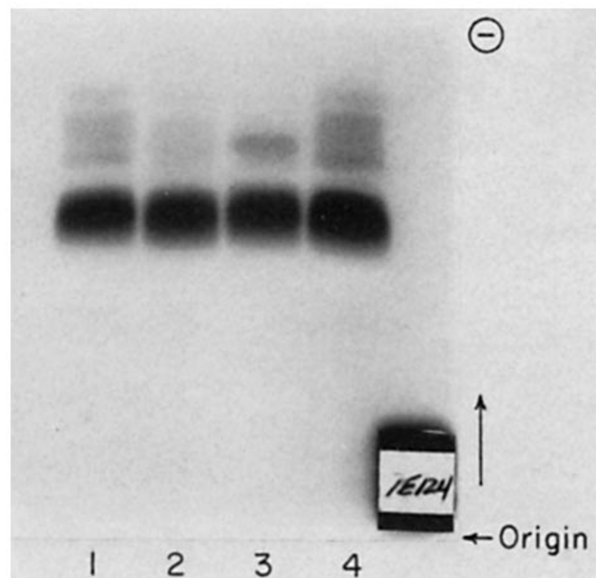

FIG. 4. Anti-DNP-polylysine antibodies and anti-DNP-BGG antibodies after dissociation.
1. Anti-DNP-BGG
2. Anti-DNP-polylysine (animal 5)
3. Anti-DNP-polylysine (animal 9)
4. Anti-DNP-BGG

other kind of pattern consisted of fewer L chain bands (Fig. 4, origin 3). In nine of ten antibody preparations examined, the bands corresponded to bands present in patterns of antibodies directed against DNP-protein conjugates.

II. Starch Gel Electrophoretic Patterns of Dissociated Antibodies Having Closely Related Immunologic Specificities.—A series of antibodies directed against cross-reacting haptens was examined by double diffusion in agar. The results were correlated with those of starch gel electrophoresis of the dissociated antibodies. The antibodies were prepared from sera of individual animals immunized with the following haptenic groups conjugated to guinea pig albumin: benzoyl, *p*-chlorobenzoyl, pentachlorobenzoyl, and dinitrophenyl. Individual sera against each of these haptens were allowed to react with all of the conju-

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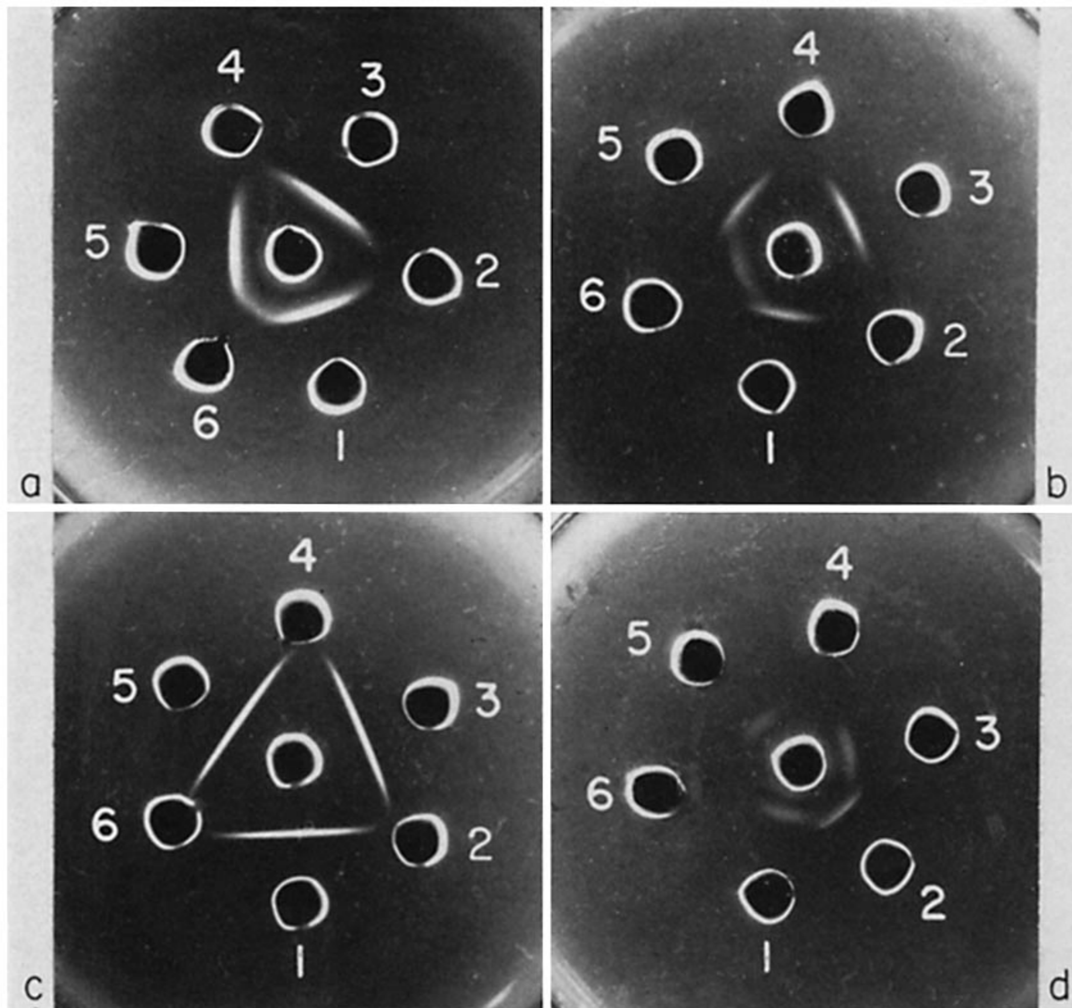

FIG. 5. Comparison of the cross-reactivity of various guinea pig antihapten antibodies by means of double diffusion in agar.

a. Central well, antibenzoyl antiserum.
1, 3, 5. B-GPA
2. DNP-GPA
4. Penta-CB–GPA
6. *p*-CB–GPA

b. Central well, anti–*p*-chlorobenzoyl antiserum.
1, 3, 5. *p*-CB–GPA
2. DNP-GPA
4. Penta-CB–GPA
6. B-GPA

c. Central well, antidinitrophenyl antiserum.
1, 3, 5. DNP-GPA
2. Penta-CB–GPA
4. *p*-CB–GPA
6. B-GPA

d. Central well, antipentachlorobenzoyl antiserum.
1, 3, 5. Penta-CB–GPA
2. DNP-GPA
4. *p*-CB–GPA
6. B-GPA

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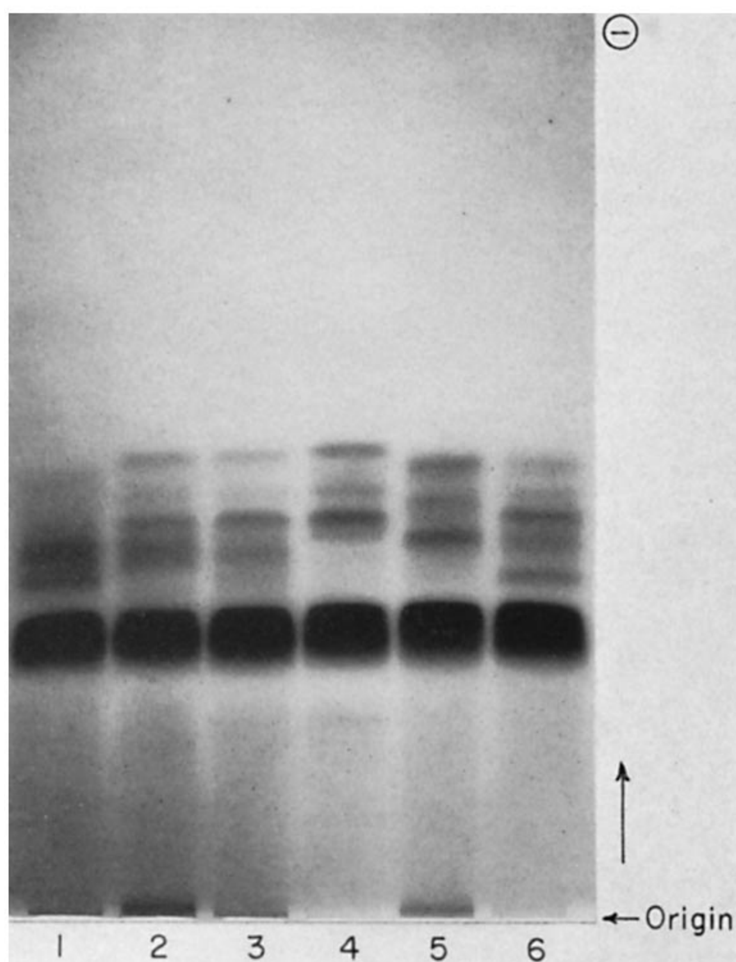

FIG. 6. Comparison of antihapten antibodies having various degrees of cross-reactivity.
1. Anti–Pic-GPA
2. Anti–B-GPA (animal 6)
3. Anti–B-GPA (animal 1-0)
4. Anti–*p*-CB-GPA
5. Anti–penta-CB-GPA (animal 1-3)
6. Anti–penta-CB-GPA (animal 2-6)

gated antigens (Fig. 5). Antibenzoyl and anti–*p*-chlorobenzoyl antibodies cross-reacted (Fig. 5, *a* and *b*) but did not form precipitin lines with DNP-GPA or penta-CB–GPA. Antipentachlorobenzoyl antibodies and anti-DNP antibodies cross-reacted (Fig. 5, *c* and *d*), particularly when antipentachlorobenzoyl antibodies were tested with DNP-GPA. Strong cross-reactivity was demon-

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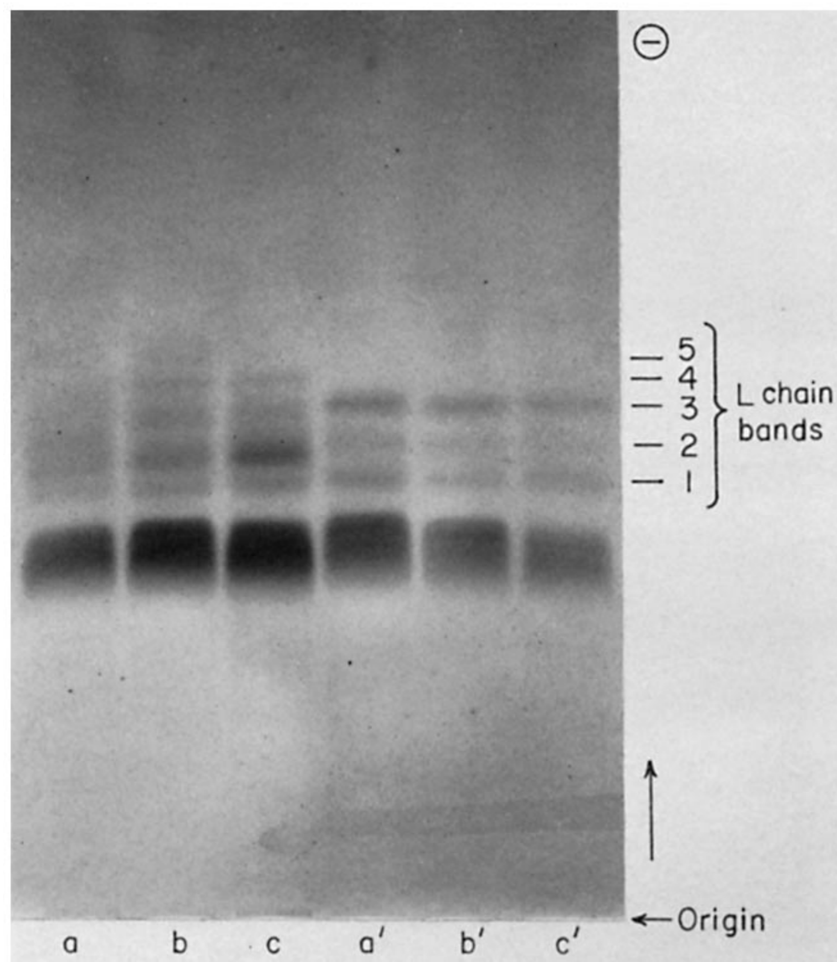

FIG. 7. Comparison of subfractions of antibodies from animals immunized with DNP-BGG.
a, *b*, *c*. DNP-lysine fractions from three separate animals
a′, *b′*, *c′*. Corresponding DNPOH fractions from the same animals
All antibodies were reduced and alkylated. Separation was by starch gel electrophoresis in urea-formate buffer.

strated only between the benzoyl and *p*-chlorobenzoyl systems, and between the dinitrophenyl and pentachlorobenzoyl systems.

The dissociated antibodies are compared in Fig. 6. Two antibenzoyl antibody preparations showed identity of four L chain bands; a fifth band was present in one of the preparations. An anti-*p*-chlorobenzoyl antibody preparation showed three bands in common with those of the antibenzoyl antibodies.

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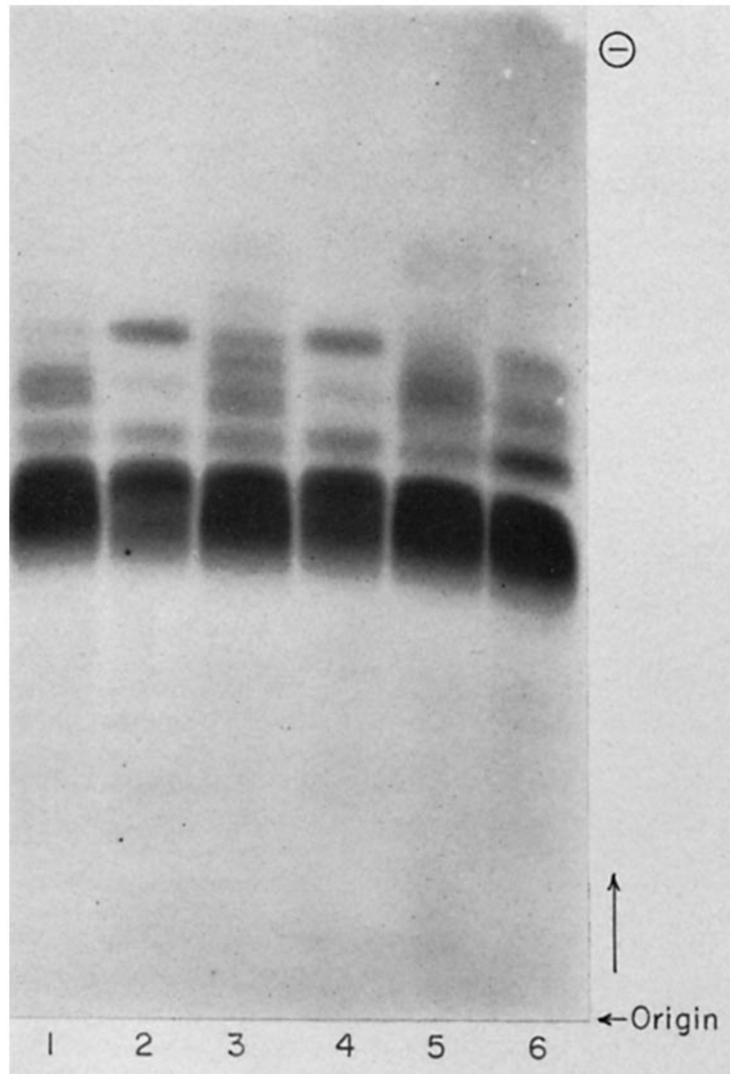

FIG. 8. Specifically isolated fractions of DNP-GPA antibodies and Pic-GPA antibodies compared after dissociation and starch gel electrophoresis.

1. DNP-lysine fraction, DNP-GPA antibodies, animal 8
2. DNPOH fraction, DNP-GPA antibodies, animal 8
3. DNP-lysine fraction, DNP-GPA antibodies, animal 1-3
4. DNPOH fraction, DNP-GPA antibodies, animal 1-3
5. DNP-lysine fraction, Pic-GPA antibodies, animal 2-1
6. DNPOH fraction, Pic-GPA antibodies, animal 2-1

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Individual antibenzoyl and anti-*p*-chlorobenzoyl antibodies had one or two bands with mobilities similar to those of antipicryl and antipentachlorobenzoyl antibodies.

III. The Effect of Specific Fractionation of Anti-DNP Antibodies on the Starch Gel Electrophoretic Patterns.—To decide whether the five L chain bands in the anti-DNP system reflected heterogeneity in the population of antibodies, the preparations were fractionated according to their degree of specificity for lysine. This is the amino acid residue in the carrier protein to which the DNP group is most frequently conjugated (9). From the precipitate of the antibodies with DNP-fibrinogen, an antibody fraction was obtained by treatment with 4×10^{-3} M dinitrophenol in 0.15 N NaCl for 16 hours at 4°C. (DNPOH fraction). After centrifugation the remaining undissolved antigen-antibody precipitate was washed, treated with 2×10^{-3} M *N*-DNP-ε-L-lysine in 0.15 N NaCl for 1 hour at 37°C, and freed of DNP-fibrinogen by addition of streptomycin (1). This fraction (DNP-lysine fraction) contained antibodies with specificities for the lysine derivative.

Comparisons of the fractions obtained from three individual anti-DNP sera are given in Fig. 7. Dissociated DNPOH fractions showed predominance of L chain bands 1 and 3; dissociated DNP-lysine fractions showed all five bands, with intense staining of band 2. To eliminate the possibility that the differences between the fractions might have resulted from small amounts of bound hapten, an excess of *N*-DNP-ε-L-lysine was added to the DNPOH fractions and an excess of dinotrophenol was added to the DNP-lysine fractions before dissociation. The characteristic patterns were uninfluenced by this procedure.

In Fig. 8 are presented the patterns of dissociated DNP-lysine and DNPOH fractions of antibodies from two additional anti-DNP sera and one antipicryl serum. The time of extraction of the DNPOH fractions was reduced to 30 minutes. As in the previous experiments, a distinct fractionation of the L chain bands was achieved.

IV. Relation of L Chains to Immunologically Active Fragments Produced by Hydrolysis of Antibodies with Papain.—The finding of different L chain patterns characteristic for antibodies of different specificities suggests that L chains might play a role in the acquisition of immunologic specificity. Experiments were therefore performed to determine whether L chains were contained in the active (S) fragments (10) produced by cleavage of antibody molecules with papain.

In a first series of experiments, antipicryl antibodies from two individual picryl-GPA sera were precipitated with picryl-microgel. After washing the insoluble precipitates with 0.15 N NaCl at 4°C, they were suspended in 0.1 M sodium phosphate buffer, pH 7.0, which was 0.01 M in cysteine and 0.002 M in versene. 0.1 mg of papain was added to the suspension and the mixture was incubated for one hour at 37°C with stirring. The suspension was then centri-

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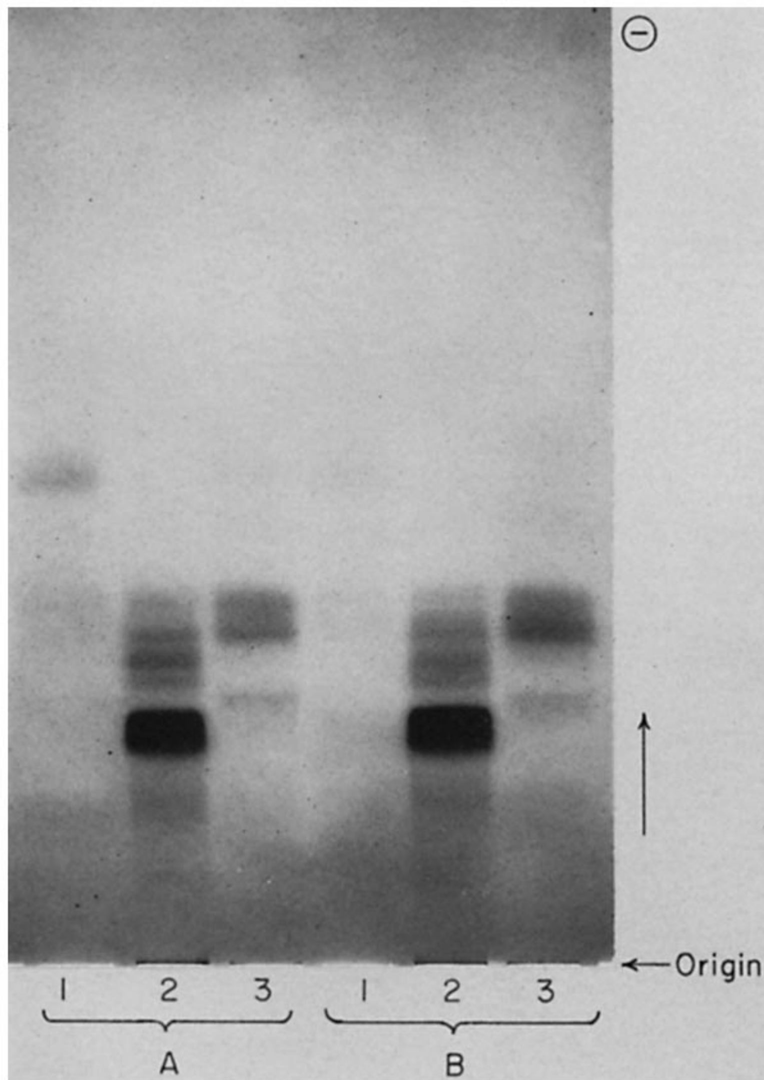

FIG. 9. Starch gel electrophoresis of active and inactive fragments of guinea pig antibodies after reduction and alkylation. The fractions obtained by papain digestion of complexes of antipicryl antibodies with picryl-microgel were reduced and alkylated and compared by starch gel electrophoresis.

1. Supernatant fraction after digestion
2. Reduced alkylated antipicryl antibodies
3. Fraction dissociated specifically from picryl-microgel

A, Guinea pig 16; B, Guinea pig 19.

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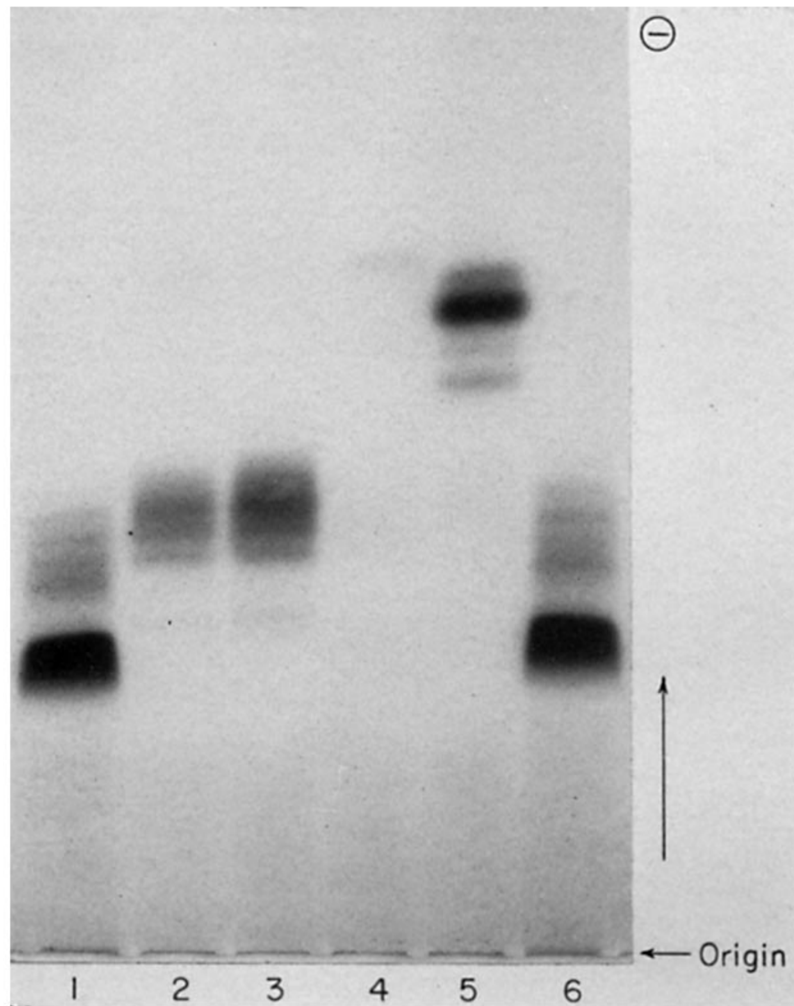

FIG. 10. Reduced alkylated fragments of papain-treated anti–DNP-lysine antibodies compared with reduced alkylated antibodies from the same animal.

1. Anti–DNP-lysine antibodies
2. Supernatant fraction of papain digest after removal of crystalline fraction
3. Supernatant fraction, chromatographic peak I
4. Supernatant fraction, chromatographic peak II
5. Crystalline fraction
6. Anti–DNP-lysine antibodies

The antibodies were obtained by pooling individually isolated anti-DNP antibodies from three different guinea pigs.

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fuged at 21,600 $\times$ G for 30 minutes and the supernate saved. The precipitate, consisting of picryl-microgel and attached protein, was washed 3 times with 0.15 N NaCl at 4°C. Univalent antibody fragments were specifically eluted by treating the washed precipitate with 1.0 ml of 2×10^{-3} M picryl-ϵ-aminocaproic acid in 0.15 N NaCl. The eluted fragments, the original supernate obtained after the papain digestion, and whole antibodies specifically isolated from the original sera were simultaneously reduced for 2 hours in 8 M urea and 0.1 M 2-mercaptoethanol. After making the solutions 0.3 M in iodoacetamide, they were compared by starch gel electrophoresis in 8 M urea (Fig. 9). The reduced alkylated antibody fragements eluted specifically from the microgel yielded bands with mobilities similar to those of the faster L chain bands of the undigested reduced alkylated antibodies. The supernatant fraction showed a band of high mobility, having no relation to any of the bands seen in the pattern of the reduced alkylated antibodies.

A second set of experiments was done on a pool of anti-DNP antibodies from three animals immunized with DNP-BGG. 75 mg of the antibody protein in 3 ml of buffer were digested with papain (see Materials and Methods). A crystalline fraction, which appeared after dialysis against distilled water, was removed by centrifugation. Chromatography of the supernatant material on DEAE cellulose under the same conditions as used previously for papain digests of human γ-globulin (10) resolved two fractions, the first of which contained the bulk of the protein. After reduction and alkylation, the entire supernatant material and the material in the first chromatographic peak showed bands, three of which corresponded to L chain bands of reduced alkylated antibodies from the original sera (Fig. 10).

After reduction and alkylation, the crystalline inactive fraction showed a fast moving group of bands that did not correspond to those of dissociated anti-DNP antibodies.

DISCUSSION

The present studies were designed to determine whether differences in immunologic specificity in a population of antibody molecules are reflected in the starch gel electrophoretic behavior of their L chains. The experiments confirm that L chain patterns of guinea pig antibodies of widely different specificities are different (1). Additional evidence has been provided that, for the most part, the differences are related to differences in specificity rather than to differences among the individual animals from which the antibodies were derived.

Varying the carrier protein to which the immunizing hapten was conjugated had little effect on the patterns of dissociated antihapten antibodies. In previous studies (1), variation of the number of haptenic groups on each carrier protein molecule was also found to be without effect. Some preparations of antibodies made against DNP-polylysine showed simpler patterns than were ob-

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tained with antibodies made against DNP-protein conjugates. It is therefore possible that within a given specificity the nature of the carrier molecule plays a part in preferentially eliciting certain antibodies.

Completely cross-reactive antibodies, *e.g.* the DNP and picryl systems, had closely similar L chain patterns. In partially cross-reactive systems of related haptens bound to the same carrier protein, the correspondences of L chains were only partial and were less uniform. Nonetheless, in almost every case some L chains of similar mobility were found. The possibility cannot be excluded that two antibody molecules with L chains of apparently identical mobility may exhibit differences in immunologic specificity. At present, there is no direct chemical evidence to indicate that each band in the starch gel corresponds to only one chemically defined type of chain. Experiments on purified myeloma proteins and Bence-Jones proteins (2, 11) suggest, however, that this may be the case.

Subfractionation of anti-DNP antibodies according to their degree of specificity for lysine indicated that the multiplicity of L chain bands in this system results from the presence of antibodies of differing specificity and structure. Heterogeneity of specificity thus appears to be reflected by structural differences in L chains even within one system of antihapten antibodies.

Several investigations (3, 12–14) have demonstrated that the antigenic determinants of L chains are on the S fragment. This fragment, which contains the antibody combining site (10, 15) yielded distinct bands after reduction and alkylation and starch gel electrophoresis. The bands had mobilities identical with those of several of the L chain bands of the reduced alkylated antibodies from which the S fragment was derived. This evidence, considered along with previous immunologic findings (12), suggests that L chains are present complete or almost complete in the S fragment. In these experiments there was no starch gel electrophoretic evidence of the H chain fragment postulated (3) to be present in the S fragment in addition to the L chain. It is possible, however, that this H chain fragment does not enter the gel or is poorly stained by amidoschwarz dye.

The foregoing results are consistent with the previously proposed notion that specificity is gained as a result of L chain–H chain interaction (3). They are also in keeping with the possibility that more than one conformation is possible for a given type of chain. It is clear that the starch gel electrophoretic findings only show a strong correlation between L chain patterns and specificity. The investigation of other chemical properties of the polypeptide chains of antibodies will be necessary in order to establish a causal connection between specificity and chain structure.

SUMMARY

Additional evidence has been obtained to show that different guinea pig antihapten antibodies differ in the structure of their L polypeptide chains. Anti-

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bodies from animals immunized with the same hapten conjugated to different carrier proteins gave similar starch gel electrophoretic patterns after dissociation of their chains. In a study of fine differences of specificity, cross-reacting antibodies were found to have some L chains with the same electrophoretic mobility. The multiplicity of L chain bands found in the characteristic starch gel electrophoretic patterns of dissociated anti-DNP antibodies was shown to be a reflection of the heterogeneity of antibodies of slightly different specificities. Reduction and alkylation of the active fragment produced by digestion of antibodies with papain yielded starch gel electrophoretic bands corresponding in mobility to L chains.

The results are consistent with the notion that L chains are involved in the acquisition of immunologic specificity.

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