*STRUCTURAL DIFFERENCES AMONG ANTIBODIES OF DIFFERENT SPECIFICITIES**

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Both the variety of specific serological reactions, and the chemical heterogeneity of the immune globulins suggest that differences in the specificity of antibodies are accompanied by appropriate differences in molecular structure. Speculation on the nature of relevant structural differences has led to two alternative assumptions: either antibody molecules of different specificity have identical primary structure and are folded differently,[1] or these antibody molecules have different amino acid sequences.[2] Up to the present, however, antibodies of the same size class but of different specificity have not conclusively been shown to differ significantly from each other, or from nonspecific γ-globulin, in any pertinent structural characteristic.

Recently, it has been found[3, 4] that γ-globulin molecules consist of several polypeptide chains that may be dissociated by cleavage of disulfide bonds. The possibility arose that this structural feature might be related to immunologic specificity. If this were so, the dissociated chains of different antibodies might show different patterns when appropriately separated. Starch gel electrophoresis in urea of reduced alkylated derivatives of γ-globulin[4] and purified antibodies was employed to investigate this possibility.

In this study, the guinea pig was chosen as a source of antibodies. This animal species could be immunized conveniently, and guinea pig γ-globulin, unlike rabbit γ-globulin,[4] could be easily dissociated by reduction. Antibodies from single animals were studied, in order to avoid the heterogeneity that might have resulted from pooling antisera. A number of antibodies directed against artificial haptens, and some antibodies specific for protein antigens, were compared by means of starch gel electrophoresis both before and after reduction and alkylation. Distinct differences were found in the electrophoretic patterns of reduced and alkylated specifically purified antibodies that were directed against different antigens.

Materials and Methods.—(a) *Animals:* Guinea pigs of either sex and of heterologous stock, each weighing 500 gm or more were used. Pre-immunization bleedings were taken in order to compare the total γ-globulin with specific antibody.

(b) *Antigens:* The following immunizing antigens were used: (1) Picryl-guinea pig albumin (Pic-GPA), preparations I, II, and III, with 28, 12, and 43 haptenic groups/mole of protein, respectively. (2) Picryl-ovalbumin (Pic-Ova), 15 groups/mole. (3) 2,4-dinitrophenyl-bovine γ-globulin (DNP-BGG), 7 groups/mole. (4) para-toluenesulfonyl-ovalbumin, (Tosyl-Ova), 22 groups/mole. (5) para-toluenesulfonyl-guinea pig albumin (Tosyl-GPA). (6) para-arsanilic-diazo-guinea pig albumin (As-GPA). (7) para-arsanilic diazo-bovine serum albumin, (As-BSA). (Antigens 5, 6, and 7 were not assayed for the number of haptenic groups conjugated to each mole of protein.) (8) Ovalbumin (Ova), 2 $\times$ recrystallized, Worthington Biochemical Corp., Freehold, N. J. (9) Bovine serum albumin (BSA), Armour Pharmaceutical Co., Kankakee, Illinois.

(c) *Other proteins:* Guinea pig albumin was prepared from pooled sera, and guinea pig γ-globulin from individual sera, by zone electrophoresis on starch.[5] Bovine fibrinogen was obtained from Armour Pharmaceutical Co., Kankakee, Illinois. Protein concentrations of the γ-globulins were determined by the modified Folin-Ciocalteu method.[6]

(d) *Immunization:* The antigens were prepared by emulsifying their solutions with an equal volume of complete Difco adjuvants containing *Mycobacterium butyricum*. They were injected initially in doses ranging from 0.4 mg to 4.0 mg distributed in 0.1 ml volumes in the four foot pads. One week to 10 days later, the antigens were injected as 0.1% solutions in 0.15 *N* saline intradermally at four sites, 0.1 ml/site. These intradermal injections were repeated every 5–7 days until severe Arthus reactivity was observed. The animals were killed 7 days after the last skin test, usually 4–6 weeks from the beginning of the immunization schedule.

(e) *Isolation of antibodies:* All guinea pig sera were first decomplemented with an irrelevant immunological system, rabbit anti-ovalbumin and ovalbumin at equivalence, except in the case of the guinea pig anti-ovalbumin sera, where equivalence precipitates of rabbit anti-bovine γ-globulin and bovine γ-globulin were used. All sera were processed individually. Anti-picryl antibodies were precipitated with picryl-bovine fibrinogen at equivalence. Precipitates were washed 3 × in cold 0.15 *N* saline, and were then dissolved in 1–2 ml of 2×10^{-3} *M* picryl-ϵ-amino caproic acid,[7] at 37°C for 30 minutes to 1 hour. 35 mg of streptomycin per ml of solution was added to precipitate the picryl-bovine fibrinogen according to the method of Farah *et al.*[8] The supernatants were dialyzed exhaustively against water and lyophilized. Anti-dinitrophenyl antibodies were isolated similarly except that DNP-bovine fibrinogen was used to precipitate, and N-DNP-ϵ-L-lysine was employed to dissociate, the antigen-antibody complexes. Anti-tosyl antibodies were also prepared in the same way. Para-toluenesulfonyl-bovine fibrinogen was used for precipitation and para-toluenesulfonyl-ϵ-amino caproic acid,[7] was the dissociating agent. Anti-ovalbumin and anti-BSA antibodies were isolated according to the method of Singer *et al.*[9] Anti-arsanilic acid antibodies were isolated using para-arsanilic-diazo-bovine fibrinogen which had been treated with picryl chloride subsequent to diazotization in order to render it precipitable by streptomycin. After routine washing of the precipitates, the antibodies were dissociated in two different ways. The soluble hapten, mono-(*p*-azobenzenearsonic acid)-chloroacetyltyrosine (kindly supplied by Dr. M. Tabachnick, Mt. Sinai Hospital, N. Y.) was used to dissociate, and streptomycin to precipitate, the fibrinogen conjugate. Alternatively, dilute H_2SO_4 solution was added to bring the pH to 2.4. Under these conditions, the antigen-antibody complex dissociated, and the fibrinogen conjugate remained insoluble.

(f) *Reduction and alkylation:* Because of the relatively small amounts of antibody available, a procedure modified from that previously described[4] was adopted. 1 to 1.5 per cent solutions of purified antibodies, or of γ-globulin, were made in 8 *M* urea, or in 8 *M* urea brought to pH 8.0 with small amounts of 1 *M* tris-(hydroxymethyl)-aminomethane buffer. Aliquots of 0.2 ml of the protein solutions were made 0.1 *M* in β-mercaptoethanol, and after 2 hours at room temperature, 12 mg of recrystallized iodoacetamide was added to each sample. After 15 minutes at room temperature, the samples were applied to the origin of the starch gel. Previous experiments indicated that the results were comparable to those obtained when the reduced alkylated samples were dialyzed before electrophoresis, provided that the samples were not allowed to stand longer than a few hours after addition of the iodoacetamide.

(g) *Starch gel electrophoresis* in formate buffer prepared with 8 *M* urea was performed as previously described.[4] All samples compared on a given gel were treated identically and simultaneously, unless otherwise specified.

(h) *Ultracentrifugation* was done using a Spinco model E ultracentrifuge with phase plate schleiren optics, and automatic temperature control. Molecular weights were measured in 6 *M* urea made 0.2 *M* in KCl, employing high speed equilibrium ultracentrifugation.[10] The partial specific volume of guinea pig γ-globulin was assumed to be 0.74.

Results.—Pooled or individual reduced alkylated guinea pig γ-globulin yielded an electrophoretic pattern consisting of a slow-moving band and a fast diffuse zone. The pattern closely resembled that previously described[4] for similarly treated human γ-globulin. In marked contrast, the patterns of reduced alkylated antibodies showed well-defined bands throughout.

In Figure 1, the patterns of pre-immunization γ-globulins and anti-dinitrophenyl antibodies are compared before and after reduction and alkylation. The untreated antibody preparations could be distinguished from the untreated γ-glob-

ulins only by their slightly different mobility. Reduced alkylated anti-dinitrophenyl antibodies yielded patterns consisting of four bands. The slowest band corresponded with that of reduced alkylated γ-globulin, and the remaining bands were distributed within the range of mobility defined by the fast diffuse zone of the treated γ-globulin. As illustrated in Figure 1, anti-dinitrophenyl antibodies from two different guinea pigs yielded similar patterns after dissociation. A total of 8 preparations from single animals were examined, and with two exceptions for which the bands were more diffuse, the patterns were nearly identical. In addition, a

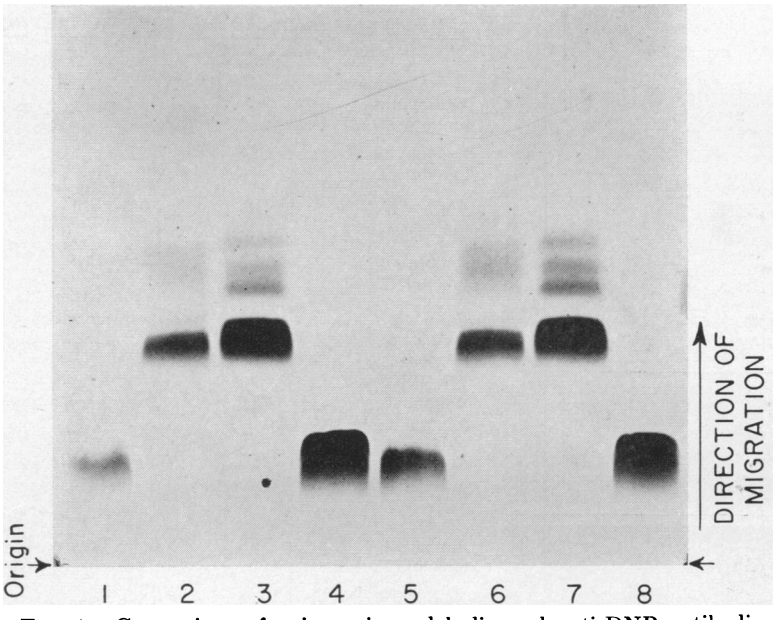

FIG. 1.—Comparison of guinea pig γ-globulin and anti-DNP antibodies before and after reduction and alkylation.
(1) Pre-immunization γ-globulin (animal ZO-4).
(2) Pre-immunization γ-globulin (animal ZO-4), reduced and alkylated.
(3) Anti-DNP antibody (animal ZO-4), reduced and alkylated.
(4) Anti-DNP antibody (animal ZO-4).
(5) Pre-immunization γ-globulin (animal ZO-5).
(6) Pre-immunization γ-globulin (animal ZO-5), reduced and alkylated.
(7) Anti-DNP antibody (animal ZO-5), reduced and alkylated.
(8) Anti-DNP antibody (animal ZO-5).

pool of anti-dinitrophenyl antibodies from four guinea pigs was reduced and alkylated and gave a pattern similar to those of individual specimens.

Reduced alkylated anti-picryl antibodies from picryl-GPA sera gave two kinds of pattern. One, consisting of four bands (Fig. 2, *f*), was barely distinguishable from that of anti-dinitrophenyl antibodies. The other pattern contained two additional fast-moving bands (Fig. 2, *e*, *h*). Of 8 anti-picryl antibody samples from individual picryl-GPA sera, 5 yielded 4-banded and 3 yielded 6-banded patterns. Three other samples from picryl-GPA sera showed more diffuse patterns, in which the bands were only faintly discernible. All of the samples obtained from four individual picryl-Ova sera yielded the 4-banded patterns. The patterns obtained

with most antibodies against tosyl-GPA were very similar to the patterns obtained with anti-DNP and anti-picryl antibodies, although slight differences were observed in the spacing of the bands.

Distinct differences were observed when reduced alkylated antibodies against certain dissimilar haptens or against protein antigens were compared in a similar fashion. Thus, anti-arsanilic acid antibodies from an As-BSA serum yielded a pattern that differed from those of anti-DNP and anti-picryl antibodies (Fig. 2, *g*). Anti-arsanilic acid antibodies from different animals showed more variable patterns after reduction and alkylation than did the anti-DNP or anti-picryl systems. Re-

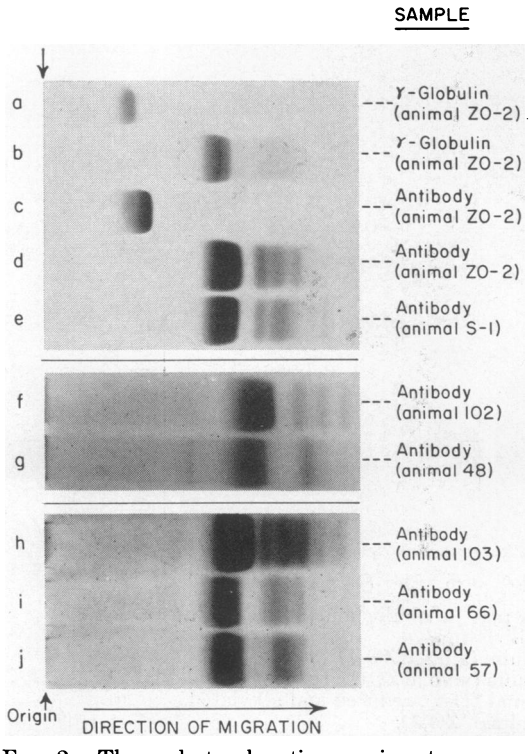

FIG. 2.—Three electrophoretic experiments comparing reduced alkylated antibodies of various specificities. *a–e*, exp. 1; *f–g*, exp. 2; *h–j*, exp. 3.

duced alkylated anti-arsanilic acid antibodies from 4 As-BSA sera showed one fast band preceded by a diffuse zone and those from 4 As-GPA sera showed 1 to 3 fast-moving bands. Similarly, antibodies directed against protein antigens showed band patterns that were slightly variable and somewhat diffuse (Fig. 2, *i*, *j*). Nevertheless, these patterns could still be distinguished from the more diffuse patterns of reduced alkylated nonspecific γ-globulin.

Further illustration of the differences in the starch gel of reduced alkylated antibodies of different specificity is given in Figure 3. The over-all similarity of the patterns of anti-DNP and anti-picryl antibodies is in contrast to the different patterns given by one particular anti-tosyl antibody preparation from tosyl-Ova serum and by two different anti-ovalbumin antibodies. Reduced alkylated anti-

BSA and anti-arsanilic acid antibodies isolated separately from the serum of the same animal immunized with As-BSA gave different patterns (Fig. 3).

Following specific isolation of the antibodies, the residual γ-globulins in the

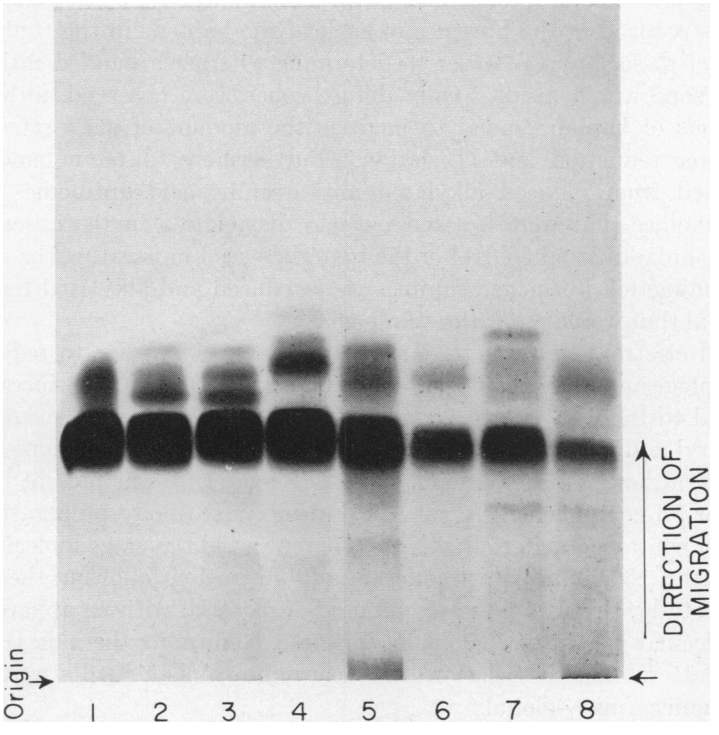

FIG. 3.—Patterns of reduced alkylated antibodies of different specificities from the same and from different animals.

(1) Guinea pig γ-globulins.
(2) Anti-DNP antibodies (animal ZO-7).
(3) Anti-picryl antibodies from picryl-GPA serum (animal 5511).
(4) Anti-tosyl antibodies, from tosyl-Ova serum (animal 4872).
(5) Anti-ovalbumin antibodies (animal 67).
(6) Anti-ovalbumin antibodies (animal 61).
(7) Anti-BSA antibodies, from As-BSA serum (animal 48).
(8) Anti-arsanilic acid antibodies, from As-BSA serum (animal 48)

Samples 1–5 reduced and alkylated at 1.5% protein concentration.

Samples 6–8 were similarly treated at a concentration of 0.75%.

The faint bands in the lower portion of the figure, below the main slow bands, appear to represent partially dissociated material the amount of which was smaller the greater the degree of reduction. The main band patterns, however, did not change with the degree of reduction.

supernatants of several antisera were electrophoretically isolated and reduced and alkylated. Their patterns on the starch gel resembled those of reduced nonspecific γ-globulin, although in most instances, a faint band just preceding the slow band was superimposed on the diffuse zone. This band, which was never seen in the reduced alkylated pre-immunization γ-globulins, may have been due to the presence of unabsorbed specific antibodies.

Most of the anti-hapten antibodies examined in this study were reduced and alkylated in the presence of a small amount of residual hapten, the last traces of which could not be removed by dialysis. The hapten conceivably might have caused the appearance of a banded pattern. Consequently, normal guinea pig γ-globulin was reduced in the presence of each of the haptens in the concentrations used to effect dissociation in order to determine whether a banded rather than a fast diffuse zone would result. Only diffuse zones were observed, although high concentrations of hapten tended to increase the amount of aggregated material resulting after reduction and alkylation. Furthermore, discrete band patterns were obtained from reduced alkylated anti-arsanilic acid antibodies and anti-protein antibodies that were isolated by acid dissociation in the absence of free hapten. As an additional control of the materials used in isolating the antibodies, variously conjugated fibrinogen samples were reduced and alkylated but failed to yield material that would enter the starch gel.

Starch gel electrophoresis of the antibody preparations prior to reduction and alkylation suggested that they consisted mainly of 7S globulin, since the zones corresponded to those of known 7S γ-globulins. Ultracentrifugal examination of one anti-picryl and one anti-tosyl antibody preparation in 1% solutions showed no 19S material, although a small amount of 9–11S aggregate was present in addition to the major 7S component. A purified anti-picryl antibody preparation from a single animal contained material with an apparent weight average molecular weight no smaller than 160,000. After reduction and alkylation following the procedure used in this study, this sample was composed of material with an apparent weight average molecular weight of 45,000 and upwards, confirming the indication by the starch gel pattern that dissociation had occurred. Similar results were obtained with whole guinea pig γ-globulin.

Discussion.—The present investigation provides experimental evidence of structural differences between specific antibodies and whole γ-globulin as well as among certain antibodies of different specificity. These differences appeared after dissociation of the antibodies into their polypeptide chains which were then partially separated by means of starch gel electrophoresis in 8 M urea. Some physicochemical properties of the dissociated chains of human and rabbit γ-globulin have been correlated with their behavior on starch gel electrophoresis in a previous study.[4] Guinea pig γ-globulin which can be dissociated by cleavage of disulfide bonds resembled dissociated human γ-globulin in its electrophoretic behavior on starch gels after reduction and alkylation. The data obtained in this study extend these observations to the dissociation of specific guinea pig antibodies.

Both normal heterogeneous γ-globulin and specifically isolated antibodies possessed slow bands of similar mobility[11] after reduction and alkylation. Instead of the diffuse fast zone shown by dissociated γ-globulin, dissociated specific antibodies exhibited well-demarcated fast bands within the range of mobility defined by this zone.

The appearance of a slow band of similar mobility in antibody and whole γ-globulin fractions suggests that γ-globulins possess one or more chains which are similar or identical from molecule to molecule. The material represented by this band had, in the case of reduced alkylated human γ-globulin, higher molecular weight than material present in the fast diffuse zone.[4] Thus, the latter material

contained subunits of lower molecular weight which varied widely in their distribution on the starch gel. In the light of the present observations, the diversity and heterogeneity of normal γ-globulin appears to be related to the large variety of chains of which any particular γ-globulin molecule might be composed.

In contrast to this continuous heterogeneity, dissociated antibodies of different specificities revealed discrete, reproducible electrophoretic patterns, which differed in the number as well as in the over-all distribution of their bands. Two highly cross-reactive antibody systems, directed against picryl and dinitrophenyl groups, yielded similar patterns. In the majority of cases, antibodies from different animals but of similar specificity possessed patterns that were alike. Variations were found, however, particularly among the anti-arsanilic acid and anti-protein antibodies. The variation seen among antibodies of similar specificity from different animals may be genetic in origin, perhaps of the kind described by Oudin.[12] In addition, these differences from animal to animal may represent differences in specificity of the isolated antibodies. It would be expected that application of detailed criteria of immunologic specificity to the intact molecules would detect finer differences among antibodies of a particular kind than the methods employed in this study. The influence of the type of protein carrier and degree of conjugation of the hapten to the carrier on the types of antibodies produced requires further definition, although no simple relationship between these factors and the starch gel patterns could be discerned when these parameters were investigated using the picryl system.

The remarkable resemblance of the sharply defined patterns of reduced alkylated purified antibodies to those of reduced alkylated multiple myeloma globulins[4, 13] possibly suggests an additional structural relationship between these abnormal proteins and the immune globulins.

The differences in the electrophoretic patterns of dissociated specific antibodies did not appear to result factitiously from the presence of the hapten during reduction and alkylation. Nor did the observed differences appear to be related to differences from sample to sample in high molecular weight (19S) antibodies. The data suggest that antibodies of different specificity consist of different types of polypeptide chains, although certain chains may be common to all the antibody molecules of a given animal. Whether the differences in electrophoretic mobility and pattern on the starch gel reflect differences in amino acid sequence or in folding cannot be decided from the present evidence. It seems likely, however, that the different chains will be found to have different amino acid compositions since separation of the extensively denatured derivatives was done in 8 M urea which would favor maintenance of the unfolded configuration. The possibility that association-dissociation interactions may occur among the subunits, even in 8 M urea, precludes the interpretation at present that a given band represents a single polypeptide chain.[14] Isolation and characterization of the chains should help to resolve this problem.

A related problem raised by the findings is whether the antibody combining site is contained within one of the subunits represented by the fast-moving bands. In this case, the multiplicity of bands observed in the electrophoretic patterns would reflect slight variations in the specificity of different antibody molecules produced against a given antigenic structure. Alternatively, the combining site may be

contributed to by the spatial arrangement of several chains. In either case, the bivalency of 7S antibody would seem to be most reasonably explained by a duplication of the relevant polypeptide chains in the molecule.

Summary.—Specifically isolated guinea pig antibodies that were reduced and alkylated, and then subjected to starch gel electrophoresis to separate their subunits yielded distinct and reproducible patterns that differed for antibodies of different specificities. Anti-hapten antibodies of similar specificity from a number of different animals possessed similar electrophoretic patterns. The patterns of reduced alkylated whole γ-globulins, although similar in some respects to those of antibodies, characteristically showed diffuse zones rather than sharp bands.

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[11] Reduced alkylated antibodies and γ-globulins electrophoresed at concentrations lower than 1% showed splitting of this slow band suggesting the possibility that two components are present.

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[14] Such interactions, if they occur, must result in stable intermediates, since 2-dimensional starch gel electrophoresis of the bands of reduced alkylated γ-globulins and myeloma proteins failed to resolve them further.

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*CHEMICAL STUDIES OF SEVERAL VARIETIES OF HB M**

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Recent studies employing the methods of X-ray crystallography have made available a rather detailed picture of the three-dimensional arrangement (the conformation) of the protein chains of sperm whale myoglobin[1, 2] and of horse hemoglobin.[3] The similarity of the conformation of the two different protein chains present in horse hemoglobin to each other and to the single chain of myoglobin has been stressed.[3] While comparable X-ray crystallographic data are not available for human adult hemoglobin, the amino acid sequence in this protein is