Isolation and Characterization of Electrophoretically Homogeneous Rabbit Antihapten Antibody Populations

II. A SURVEY OF THE ISOELECTRIC PROPERTIES OF ANTITHAPTEN ANTIBODIES DIRECTED AGAINST CHARGED AND UNCHARGED HAPTENS*

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SUMMARY

Liquid isoelectric focusing profiles of a number of conventionally raised rabbit antibodies directed against acidic, neutral, and basic haptens have been determined. All antibody preparations showed a degree of heterogeneity similar to that already reported for antibodies to the basic hapten p-azophenyltrimethylammonium. There was little difference in the degree of heterogeneity found in the focusing profiles of antibody from different rabbits directed against the same hapten. There appear to be between 10 and 30 individual antibody species in each case, but some of this complexity may be a result of variable, postsynthetic deamidation. Isoelectric focusing profiles of the antigens also showed considerable heterogeneity. The mean isoelectric point of both rabbit and bovine γ-globulins was approximately pH 6.0, and the distribution of the isoelectric point, pI, values of the focused specifically purified rabbit antibodies about this point was reciprocally related to the pI of the focused antigens. It appears that a single charge unit causes a displacement of the mean pI value by approximately 0.1 pH unit. The displacement found for various antibody preparations indicates antibodies to acidic and basic haptens may differ by as many as 19 charged residues.

The heterogeneity present in antihapten antibodies purified on immuno-absorbents (1, 2) results from the nature and heterogeneity of the antigen (3, 4), the route and duration of the immunization, and certain genetic factors in the animal being immunized (5). Pressman, Boholt, and Grossberg (6) suggested that the heterogeneity of antibodies need not be as complex as the electrophoretic pattern or binding plot at first appears to indicate. There are, however, few procedures which permit further analysis of the heterogeneity of antibody preparations.

We have previously reported the use of preparative liquid isoelectric focusing (7, 8) for the further fractionation of specifically purified antibodies directed against the basic hapten Ap1 (p-azophenyltrimethylammonium) (9). Some of the better resolved components displayed properties which are characteristic of individual myeloma proteins and of homogeneous antibodies. The technique appears to be a promising one for the examination of the degree of heterogeneity in preparations of specifically purified antibody and also as a means for characterizing the isoelectric properties of antibodies directed against variously charged and uncharged haptens and antigens.

Although Krause (10) and Haber (11) have individually reported that very simple mixtures of antibodies against some bacterial polysaccharide antigens can be produced under certain immunizing conditions, we have chosen, as a first step, to examine antihapten antibody raised in a conventional manner, with Freund’s adjuvant, to determine the degree of heterogeneity present under these circumstances. In this paper we report the isoelectric focusing profiles of antibodies directed against a number of neutral, negatively and positively charged haptens.

The haptens used in this study, coupled to BGG, were chosen so as to represent typical acidic, neutral, and basic groups. Sela and Mozes (12) and Rude, Mozes, and Sela (13) have made a study of the charge properties of antibodies directed against antigens of differing charge. With the chromatographic behavior on DEAE-Sephadex of various antihapten antibodies as an index of their over-all charge, they demonstrated, in a number of

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species, a reciprocal relationship between the net electrical charge on the antibody and the charge on the antigen as a whole. These findings have been confirmed by Knight, Roelofs, and Haurwitz (14) and Benacerraf et al. (15). However, Stylos, Merryman, and Maurer (16) and Maurer, Merryman, and Stylos (17) have reported that in sheep, a species not included in previous studies, this relationship does not appear to hold true. We have used isoelectric focusing to measure not only the distribution of isoelectric points in the antibodies but also the modification in charge of the antibody and the immunogen noted by Sela and Merryman, and Maurer (16) and Maurer, Merryman, and Stylos (18) have been able to measure the charge properties of the antigens and of the antibodies which they elicited and have confirmed that in the rabbit, the reciprocal relationship between the net electrical charge of the antibody and the immunogen noted by Sela and Moses (12) and Rude et al. (13) holds true.

**EXPERIMENTAL PROCEDURE**

**Preparation of Antigens—Azoproteins were prepared by coupling bovine γ-globulin (BGG, Pentex) with diazotized p-aminophenyltrimethylammonium chloride (Eastman) to give BGG-AP (18), with diazotized 3-aminopyridine (K and K Laboratories, Plainview, New York) to give BGG-P3 (18), with diazotized p-aminobenzoate (Fisher) to give BGG-Xp (18), 19), with diazotized 6-aminonaphthalene 2-sulfonate (Eastman) to give BGG-aNS (20), with p-phosphanilate (prepared by Dr. V. P. Kreiter, Spring Valley, New York) to give BGG-Pp (21) and with diazotized p-azobenzeneamine (anti-aNS) (19), p-azobenzeneamine (anti-Ap) (18), 3-azopyridine (anti-Ap) (18), p-azobenzoate (anti-Xp) (18), 6-azonaphthalene 2-sulfonate (anti-aNS) (19), p-azobenzene-phosphonate (anti-Pp) (20), and p-azobenzene-sarboxonate (anti-Rp) (18).

**Preparation of Antisera—**The methods have been previously described for preparing and testing rabbit antisera directed against p-azophenyltrimethylammonium (anti-Ap) (18), 3-azopyridine (anti-P3) (18), p-azobenzoate (anti-Xp) (18), 6-azonaphthalene 2-sulfonate (anti-aNS) (19), p-azobenzene-phosphonate (anti-Pp) (20), and p-azobenzene-sarboxonate (anti-Rp) (18).

**Purification and Characterization of Antihapten Antibodies—**The immunoglobulin fractions of rabbit antiapten antisera and normal rabbit immunoglobulins (RGG) were prepared by three sodium sulfate precipitations at room temperature (22). Specifically purified antihapten antibodies were prepared by a single precipitation of the antisera with 50% saturated ammonium sulfate at 4°C, followed by adsorption on a specific solid immunosorbent (made by coupling mercaptosuccinyl groups to rabbit serum albumin) and then cross-linking the modified protein with tris(1,2-ethylenediimid)phosphine oxide (23, 24). The adsorbed antibodies were eluted with 0.02 M glycine-HCl buffer, pH 2.3 (24), or with 1 M propionic acid (containing 0.1 M NaCl) at 4°C (25). The antibodies were dialyzed against Tris-Cl buffer, pH 8.0, and concentrated by pressure ultrafiltration (26). The specifically purified antibody preparations were calculated to contain more than 95% antibody as determined by equilibrium dialysis with [14C]labeled haptens (27). Specifically purified anti-Ap (Rabbit 2284 and 4240), anti-P3 (Rabbit 3781), anti-Rp (Rabbit 4278), anti-aNS (Rabbit 5159 and 5158), and anti-NIP (Rabbit 5159) antibodies were kindly supplied by Dr. A. Grossberg (Roswell Park, Buffalo, New York).

**Preparation of Human Myeloma Proteins—**Human Ig myeloma proteins (McC and Nub) were purified from serum by Porath column electrophoresis (Tris-borate buffer, pH 8.6) followed by gel filtration on Sephacry G-200 (10.0 × 100 cm) in 0.3 M glycine. The purity of the myeloma proteins was checked by both cellulose acetate electrophoresis, pH 8.6, and by borate starch gel electrophoresis, pH 8.6.

**Treatment of Antibody and Myeloma Proteins to Remove Amide Ammonia—**Samples of myeloma proteins were incubated at pH 7.0 with t-asparaginase (Worthington) (28) or held at 37°C for 72 hours in pH 3.0 acetate buffer or pH 10.0 glycinate buffer in the presence of 0.02% sodium azide (29). Success in conversion to a common electrophoretic species was evaluated by starch gel electrophoresis.

**Assay of Amide Groups—**Methods used to measure the relative amide content of isoelectrically isolated antibodies included the following. Liberated ammonia following mild acid hydrolysis in 1 N H2SO4 at 100°C for 60 min was measured by Conway microdiffusion (30, 31); amino acid analysis, with careful measurement of adventitious ammonia; determination of free carboxyl groups by carbodiimide reaction (32) with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide-HCl (Ott Chemical Company, Muskegon, Michigan) and glycine methyl ester-HCl (Cyclo Chemical Company, Los Angeles, California).

**Treatment of Anti-Ap Antibody to Remove Sialic Acid—**Samples of antibody (16 mg) were dissolved in sodium acetate buffer, pH 4.8, at a concentration of 8 mg per ml and were incubated with 0.2 ml of neuraminidase prepared from Clostridium perfringens (a gift of Dr. H. Schachter, University of Toronto). The neuraminidase had been shown to be capable of releasing 0.12 μmol of sialic acid per ml of orosomucoid per min and was shown to be free of proteolytic activity by incubation with Azocoll (Calbiochem) overnight. The mixture was incubated at 37°C for 60 min and held frozen until required for isoelectric focusing. Free sialic acid was measured by the thiobarbituric acid method (33) and the total sialic acid was measured by the resorcinol-HCl method (34, 35).

**Fractionation of Antihapten Antibody Preparations—**Samples of specifically purified antihapten antibodies, from individual rabbits, were fractionated by liquid isoelectric focusing with the LKB 8101 (110 ml) electrofocusing column (LKB Instruments, Inc., Stockholm, Sweden) as described by Yesterberg and Svensson (8) and incorporating all of the modifications of Freedman and Painter (9). The focusing columns were run without the protein samples at a constant voltage of 1000 volts for 24 hours at 3°C. Then the antibody preparations were added (9), and the samples were electrofocused in the usual manner for 24 to 72 hours at a constant voltage of 1000 volts. Blank focusing columns were run at pH 3 to 10 and 5 to 8 for the same time and at the same voltage as the corresponding antibody runs, to establish the correct base-lines for the antibody focusing profiles. pH measurements were made with a Radiometer PHM 260 pH meter at 3°C immediately after the proteins were eluted from the column. Protein measured in pooled major fractions represented between 65 and 85% of the sample applied. Estimates of the areas of the traces indicate that all the sample applied can be accounted for in the absorbance trace.

**RESULTS**

**Comparison of Isoelectric Focusing Profiles over Narrow Range (pH 5 to 8) of Anti-Ap and Anti-Rp Antibodies—**Examples of the isoelectric focusing patterns, over the pH range 5 to 8, of the
Isoelectric Properties of Rabbit Antihapten Antibodies

Fig. 1. The liquid isoelectric focusing elution profiles of 12 mg of specifically purified a, rabbit 4240 anti-Ap antibodies, b, rabbit 1397 anti-Ap antibodies, and c, rabbit 4810 anti-Ap antibodies in the pH 5 to 8 gradient range at 1000 volts for 72 hours, with the use of the 110-ml LKB electrofocusing column. ---, pH gradient; --, continuous absorbance at 280 nm, ---, the absorbance trace of a control column run in the absence of protein.

Fig. 2. The liquid isoelectric focusing elution profiles of 12 mg of specifically purified a, rabbit 2828 anti-Rp antibodies, b, rabbit 4278 anti-Rp antibodies, c, rabbit 6277 anti-Rp antibodies, and d, rabbit 4183 anti-Rp antibodies in the pH 5 to 8 gradient range. Conditions are identical with those outlined in Fig. 1. The hatched line represents a change of absorbance scale to 0 to 3. The dashed, solid, and dotted lines are the same as in Fig. 1.

antibody purified from three rabbits immunized with the basic hapten Ap diazotized to BAG are shown in Fig. 1. It can be seen that in all cases a profile of similar complexity was obtained. The pattern shows two main groups of components, one focusing at the basic end of the pH gradient and containing three or four well resolved components which correspond with those whose properties have already been examined in detail (9). The other main component focuses at lower pH values and is less well resolved under these conditions. Within this pattern may be recognized individual variations both in concentration and isoelectric point, pH, values which are characteristic of the individual rabbits.

The isoelectric focusing profiles, over the pH range 5 to 8, of antibody directed against an acidic hapten Rp from four rabbits are shown in Fig. 2. This antibody preparation gave a pattern of similar complexity to that given by anti-Ap antibody (Fig. 1). There appears to be more variation between individual rabbits, there being no over-all pattern which may be recognized as common to all rabbits, as was the case for Ap. In all cases the antibodies are distributed over the pH range 5.5 to 8.0 which is somewhat displaced from the range in which anti-Ap antibodies focus, namely pH 5 to 7. This point will be explored in more detail below. There are a number of well resolved components in all preparations of anti-Rp antibody. The heterogeneity is typical not only of all patterns seen with antibodies to these two antigens but also of specifically purified antibodies directed against the other haptenes reported in this study (Pp, Pα, Xp, and aNS). None of these antihapten antibodies gave rise to less complex patterns.

Number of Different Antibodies Comprising Each Preparation—From Figs. 1 and 2 it might be inferred that the minimum number of individual antibodies present in each of these preparations is...
Fig. 3. The liquid isoelectric focusing elution profiles of 10 mg of purified a, MeC. myeloma protein and b, NuB. myeloma protein in the pH 7 to 10 gradient range. Conditions identical with those outlined in Fig. 1. The dashed, solid, and dotted lines are the same as in Fig. 1.

Fig. 4. The liquid isoelectric focusing elution profiles of 12 mg of specifically purified a, rabbit 2277 anti-Xp antibodies, b, rabbit 5158 anti-AN antibodies, c, rabbit 1738 anti-Pp antibodies, and d, rabbit 8740 anti-Rp antibodies in the pH 3 to 10 gradient range. Conditions identical with those outlined in Fig. 1. The hatched line represents a change of absorbance scale to 0 to 3. The dashed, solid, and dotted lines are the same as in Fig. 1.

Fig. 5. The liquid isoelectric focusing elution profiles of 12 mg of specifically purified a, rabbit 4240 anti-Ap antibodies, b, rabbit 3743 anti-P3 antibodies, c, rabbit 2284 anti-Ap antibodies, and d, rabbit 5689 anti-P3 antibodies in the pH 3 to 10 gradient range. Conditions identical with those outlined in Fig. 1. The hatched line represents a change of absorbance scale to 0 to 3. The dashed, solid, and dotted lines are the same as in Fig. 1.

About twenty. However, the number of distinctive antibodies may not be nearly as great as this. Other sources of heterogeneity than antibody specificity may be present. For example microheterogeneity in the carbohydrate moiety of each antibody could give rise to a number of peaks in the isoelectric focusing profile from the same antibody. Similarly, postsynthetic deamidation might result in each antibody being present in a number of electrophoretically distinguishable forms. These would correspond to the allomeres which Johnson and Deutsch (36) have identified in the Fab fragment of certain myeloma proteins. In Fig. 3 the isoelectric focusing profile of two typical human myeloma proteins is shown. That shown in Fig. 3a is a myeloma protein which focuses as a single homogeneous component. That seen in Fig. 3b, however, is composed of a series of components, each present in decreasing concentration towards the acidic end of the gradient and spaced approximately 0.1 pH unit apart. It is thought that these components differ only in their content of dicarboxylic acid and amide groups (37). If each antibody in Figs. 1 and 2 were present in a similar degree of heterogeneity as the protein in Fig. 3b, the number of different antibodies might be considerably less than the number of peaks seen in Figs. 1 and 2.

To determine whether microheterogeneity in the carbohydrate content of each antibody was contributing to the complexity of the focusing pattern the following experiment was carried out. Samples of anti-Ap antibody were divided into two equal parts. One part was treated with neuraminidase to remove the sialic
acid and then both samples were focused individually in separate focusing columns under identical conditions. While the relative amount of each component differed somewhat in the two patterns, the general distribution of protein and the over-all number of components was not altered by the removal of the sialic acid. In this experiment an average of 1 residue of sialic acid per molecule of immunoglobulin was removed by neuraminidase. The same experiments were performed with anti-Pp and anti-NIP antibody with the same results.

Similar experiments were attempted in order to determine the contribution of carboxyl groups, arising from postsynthetic deamidation of each species of antibody, to the heterogeneity of the pattern. Acid hydrolysis for 60 min at 100° with 1 N HCl was used. This period was previously established as the minimum time under which a maximum yield of ammonia was produced under these conditions, although ultracentrifugal analysis and starch gel electrophoresis indicated that it resulted in considerable degradation of the protein. The focusing pattern produced was found to be considerably displaced toward the acidic end of the pH gradient (pH 2.0 to 6.0) and was even more complex than the untreated sample. It was not possible to determine whether there was any simplification in the pattern as a result of deamidation, because further heterogeneity was probably introduced as a result of peptide bond cleavage. Attempts to achieve deamidation by enzymic (L-asparaginase) and nonenzymic means (pH 3 or 10 incubation) were not successful. The electrophoretic patterns obtained after these treatments closely resembled the patterns obtained with untreated material.

Table I

| Hapten* | Rabbit No. | Area p\% of whole pI values Estimated mean pI |
|---------|------------|---------------------------------------------|
| Rabbit γ-globulin | | | |
| Basic | | | |
| Ap | 4210 | 70 | 30 | 5.8 |
| Ap | 2284 | 65 | 35 | 5.5 |
| Ap | 4810 | 60 | 40 | 5.9 |
| Ap | 1397 | 62 | 38 | 5.5 |
| Ap | 4278 | 70 | 30 | 5.7 |
| Acidic | | | |
| Rp | 2828 | 7 | 93 | 7.2 |
| Rp | 6277 | 8 | 92 | 6.9 |
| Rp | 4278 | 6 | 94 | 7.0 |
| Rp | 8743 | 13 | 87 | 6.3 |
| Rp | 4183 | 15 | 85 | 6.8 |
| aNS | 5156 | 15 | 85 | 7.4 |
| aNS | 5158 | 15 | 85 | 7.4 |
| Xp | 2277 | 22 | 78 | 6.5 |
| Pp | 1738 | 30 | 70 | 6.3 |
| Neutral | | | |
| P3 | 3743 | 48 | 52 | 6.0 |
| P3 | 5680 | 46 | 54 | 6.2 |

* Hapten was diazotized to BGG for use as an antigen and the antibodies were specifically purified by the use of the same hapten coupled to a solid immunoadsorbent.

Areas of the isoelectric focusing profile on either side of pH 6.0 were measured by planimetry and are expressed as a percentage of the total area.

Estimated mean pI values were estimated by eye and tested by planimetry.

Correlation between Isoelectric Properties of Antigen and Antibody—It was noted above that the antibodies produced in response to basic haptens focus in the acidic end of the pH gradient within the range pH 5 to 8 (Fig. 1), whereas antibodies produced in response to acidic haptens tend to be distributed at the basic end of the pH gradient (Fig. 2). To investigate this more fully, and to include all the antibody proteins in the focusing pattern, isoelectric focusing profiles of antibody were prepared over the pH range 3 to 10. Typical focusing profiles over the range pH 3 to 10 of antibodies to a number of different haptens coupled to BGG are shown in Figs. 4 and 5.

Fig. 4 shows typical isoelectric focusing profiles over the range pH 3 to 10 of specifically purified antibodies directed against acidic haptens. In all cases most of the protein was located in the pH gradient above pH 6. The estimated pI values for these antibodies are shown in Table I. The displacement from pH 6.0 varies from 0.3 for anti-Pp antibody to 1.4 for anti-aNS antibody. All antibodies show a similar degree of complexity which, of course, may be resolved further by focusing over a narrower pH range (Figs. 1 and 2). In Fig. 5 are shown the isoelectric focusing profiles over the range pH 3 to 10 of RGG and BGG. In both cases the protein is distributed evenly about a mean pI value of 6.0 over a wide range of pH, approximately pH 3 to 10. Thus, the antibodies directed against acidic haptens are found in the gradient toward the basic end of the distribution pattern of the
normal immunoglobulin population. Fig. 5 shows typical isoelectric focusing profiles in the pH range of 3 to 10 of antibodies directed against basic and neutral haptens. The majority of the protein in the isoelectric focusing profile of anti-Ap antibody (Fig. 5, a and c) focused below pH 6 while in the focusing profiles of antibodies to the neutral hapten Ps (Fig. 5, b and d), the antibody distribution curve resembles that of the whole rabbit immunoglobulin in that it is centered about pH 6. The estimated mean pI values for these antibodies are shown in Table I. The displacement from pH 6.0 of the mean pI of anti-Ap antibodies (0.1 to 0.5) is smaller than that of antibodies directed against acidic haptens.

In Table I, the areas of the isoelectric focusing profile on either side of pH 6 are expressed as a percentage of the whole area. It can be seen that not only is the mean pI displaced but the whole population of proteins comprising the antigen is displaced above or below pH 6.0 depending on whether the hapten is basic or acidic. BGG-Ap (Fig. 7a) is distributed over the pH range 5 to 10 with an estimated mean pI of 7.8 and BGG-Rp is distributed over the range 3 to 7 with a mean pI of 4.9 (Fig. 7b).

**DISCUSSION**

The use of liquid isoelectric focusing for the further resolution and analysis of anti-hapten antibodies has allowed a quantitative evaluation of the complexity of antibody mixtures. The same type of analysis was also applied to the antigens. A comparison of the results of the analyses of both antigens and antibodies permitted a quantitative evaluation of the relationship between the net electrical charge on the antigen and the antibody which it elicits.

The carrier protein for the antigens was, in all cases, BGG. The isoelectric focusing profile of unmodified BGG showed the preparation to be uniformly distributed about a mean pI of 6.0 (Fig. 6b) indicating that it may be regarded as an essentially neutral carrier. The degree of the displacement of the pI as a result of hapten coupling varied with the hapten. The greatest displacement of the mean pI for the acidic haptens occurred with aNS which caused the pI of BGG to be displaced from 6.0 to 3.5 (Table II). The least displacement for the acidic haptens occurred with Rp which displaced the pI of BGG by approximately 1.1 pH units (Table II). The basic hapten (Ap) shifted the pI of

| Antigen       | Mean pI values | Estimated mean pI |
|---------------|----------------|-------------------|
| Bovine γ-globulin |                |                  |
| Basic         | 7.8            |                  |
| Acidic        |                |                  |
| BGG-Rp        | 4.9            |                  |
| BGG-Pp        | 4.0            |                  |
| BGG-Xp        | 3.6            |                  |
| BGG-aNS       | 3.5            |                  |
| Neutral       |                |                  |
| BGG-P3        | 5.8            |                  |

* Areas of the isoelectric focusing profile on either side of pH 6.0 were measured by planimetry and are expressed as a percentage of the total area.

* Mean pI values were estimated by eye and tested by planimetry.
BGG by 1.8 pH units (Table II). Among the factors determining the extent of displacement will be the degree of substitution by hapten and the pK of the substituent group. In general, it appears that the introduction of about 20 charge residues onto the BGG molecule resulted in a displacement of about 2 pH units. Thus each charged residue caused a displacement of about 0.1 pH unit in this region of the pH scale.

The isoelectric profiles of the antigens revealed a considerable degree of heterogeneity in the antigen preparations. This undoubtedly is one of the major factors in the heterogeneity of the antibody response to these antigens. The virtue of using single antigens to obtain antibodies of restricted heterogeneity has been shown by a number of investigators (3, 4, 10, 11). The use of preparative isoelectric focusing should permit the isolation of useful quantities of antigens of considerably reduced heterogeneity.

The isoelectric properties of the antibodies elicited by the variously charged antigens may be seen in the isoelectric focusing profiles of the specifically purified antibody preparations over the pH range 3 to 10 (Figs. 4 and 5). Antibody to acidic antigens focused in the basic region of the pH gradient whereas antibody to basic antigens focused at the acidic end of the pH gradient (Table I).

Flatmark (38) has shown that the shift in the pI of cytochrome c caused by a single charge unit, at a pH above 10, is of the order of 0.2 pH unit. An examination of the distribution of the several components of myeloma proteins which focus like that shown in Fig. 3b indicates that these components are spaced 0.1 pH unit apart in the pH gradient over the range 4 to 9. This is consistent with the observations made above that the introduction of a single charge haptenic group onto BGG caused a displacement of 0.1 pH unit. The difference between our observations for myeloma proteins and antigens and those of Flatmark for cytochrome c may be a result of the buffering in the pH scale below pH 10 (e.g. the imidazole group at pH 6.0) and the total number of charged residues on the molecule.

The displacement of pI of antibodies directed against charged haptens may be used to estimate the differences in the number of charged residues in these different populations of antibodies. Thus, the maximum displacement of 1.9 pH units (Table I) between antibodies directed against acidic and basic antigens indicates that these antibodies differ by about 19 charge residues. This result is consistent with the finding (9) derived from the over-all amino acid analyses of the focused anti-Ap antibody fractions. Focused Fraction A, with a mean pI of 7.2, contained an over-all charge of -114 while focused Fraction D, with a mean pI of 5.8, contained an over-all charge of -125. Assuming that the glutamic acid and aspartic acid residues in these antibodies were substituted as amides to a similar extent, the over-all charge content of the focused fractions was quite consistent with the concept that a displacement of 0.1 pH unit represents a difference of 1 charge unit. Amino acid analyses of antibodies directed against acidic (Rp) and basic (Ap) haptens have been shown by Koshland and Engleberer (39) and Koshland, Engleberger, and Shapanka (40) to differ by 10 charge units. In our case, we found that the differences in mean pI between anti-Ap and anti-Rp antibodies were 0.8 pH unit reflecting approximately 8 charge units.

This number of charged residues is more than might be expected to be involved in direct interaction between antibody and the haptenic group. This is consistent with the general hypothesis that the initial reaction between antigens and the receptors on immunocompetent cells is based on their over-all charges being complementary. Sela et al. (41) and Sulica, Tarrab, and Sela (42) for example, showed that the removal of acidic cells reduced the capacity of a spleen population to respond to basic antigens. The inverse relationship between the pI values of the antigen and the antibody (IgG) depends on the net electric charge of the intact immunogen and not on the net charge within a limited area around the antigenic determinants (13, 41). Mozes, Robbins, and Sela suggested that the differentiation between antibodies directed against charged antigens derives primarily from the light (43) and not the heavy (Segal, Givol, and Sela (44)) chains. We are currently studying the variable N-terminal sequences of the focused anti-Ap and anti-Rp antibody light chains in collaboration with Dr. W. Terry (National Institutes of Health). Of all the antibodies directed against acidic haptens anti-Rp antibody appeared to be the most suitable for further study because of the ease with which the many components were resolved (Fig. 2). Antibody directed against the Ap hapten yielded isoelectric focusing profiles containing the largest number of individual components (Fig. 1) and preliminary sequencing of these antibodies has already been reported (9).3

The results presented in this paper also show that within the number of rabbits studied, there is no marked variation in the response of individual rabbits to a specific antigen in terms of the complexity of the isoelectric focusing pattern of the specifically purified antibodies. All antibody preparations examined showed a considerable degree of complexity with 10 to 30 components present in each. This heterogeneity was not surprising, since most of the rabbits were immunized by the use of multiple intradermal injections with Freund's adjuvant and with antigens known to be heterogeneous. Further heterogeneity may have been introduced by pooling samples from a number of bleedings from each rabbit (45) although recent work (46) suggests that this might not increase the heterogeneity. In the previous paper in this series (9), it was shown that well resolved components in these patterns displayed properties consistent with their being homogeneous antibodies. Thus, examination of the profiles of anti-Ap and anti-Rp antibodies focused over a narrow pH range, 5 to 8 (Figs. 1 and 2) indicates that each rabbit, during the course of its immunization, may have produced as many as 20 to 30 electrophoretically distinguishable antibodies to each antigen.

We have reason to suspect, however, that the number of individual antibodies may be fewer than this. Awdeh, Williamson, and Askonas (37) have shown that monoclonal myeloma proteins exhibit polydisperse behavior in electrophoretic systems and that this develops progressively following biosynthesis. This is seen in the focusing profile of the myeloma protein in Fig. 3b. It has been suggested that this heterogeneity is a result of either variable glycosylation of identical peptide chains (37), variable removal of carbohydrate components (36), or variable post-synthetic conversion of amide groups to free carboxyls (37). Robinson, McKerrow, and Cary (47) suggest that post-synthetic deamidation is a common phenomenon and that it is associated with the aging of serum proteins. The isoelectric focusing profiles of two human myeloma proteins are shown in Fig. 3a and b. The heterogeneous pattern seen in Fig. 3b is characteristic of many myeloma proteins and is thought to result from heterogeneity in either car-

1 M. H. Freedman, unpublished results.

2 W. D. Terry and M. H. Freedman, unpublished results.
bohydrate or amide groups on the proteins. Similar multiple forms have also been described in Fab fragments and called allomeres (30). This term will be used here to describe molecules derived from the same biosynthetic source and modified in some way following synthesis. If any of the antibodies were present in a large number of allomorphic forms, each peak in the focusing profile need not necessarily represent an individual antibody. Thus a pattern of the complexity of those seen in Figs. 1 and 2 might be produced by as few as three or four antibodies if they were of the type seen in Fig. 3b. This accords well with the suggestion put forward by Pressman, Roholt, and Grossberg (6) but attempts to show this have so far met with little success.

It has not been possible to measure accurately the number of free carboxylic acid residues or the number of amide groups in each of the separated components of the myeloma proteins, such as that seen in Fig. 3b. The error of the methods which may be used is invariably greater than the size of the difference which must be measured in order to detect a single charge difference, since this is all that is likely to distinguish the proteins making up the type seen in Fig. 3b. This accords well with the suggestion put forward by Pressman, Roholt, and Grossberg (6) but attempts to show this have so far met with little success.

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