Isolation and Characterization of Electrophoretically Homogeneous Rabbit Antihapten Antibody Populations

I. SEPARATION AND PROPERTIES OF HOMOGENEOUS ANTI-\textit{p}-AZOPHENYLTRIMETHYLAMMONIUM ANTIBODIES*

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SUMMARY

A method is described by which liquid isoelectric focusing over a pH range 5 to 8 has been used on a preparative scale for the further purification of specifically purified antibodies directed against the positively charged \textit{p}-azophenyltrimethylammonium hapten (anti-Ap antibodies). The anti-Ap antibodies separated into 4 to 8 major fractions and 10 to 16 minor fractions. More than 95\% of the antibodies directed against the positively charged hapten had isoelectric points between pH 5 and 8. The charge, structural, and functional homogeneity of several of the major fractions was assessed by a number of independent criteria. The charge homogeneity of three of the electrofocused antibody fractions was demonstrated by the presence of monodisperse zones in electrophoresis, by focusing the major fractions over a narrow pH range, and also by acrylamide disc electrophoresis of fully reduced and alkylated light chains in alkaline urea gels. The structural homogeneity of two of the electrofocused antibody fractions was demonstrated by the finding of single NH$_2$-terminal residues on the isolated, fully reduced and alkylated light chains. Sequence studies on the isolated light chains of these fractions showed each to have a single coherent sequence compared with the parent antibody which was typically heterogeneous. These antibody fractions showed only the b$_4$ allotypic marker on their light chains and 95 to 99\% of the heavy chains contained the a$_1$ allotypic marker, although the unfragmented antibodies were heterozygous (a$_1$, a$_3$) with respect to the heavy chain allotypic markers. The functional homogeneity of these electrofocused antibody fractions was determined by equilibrium dialysis using the $^{125}$I-labelled \textit{p}-iodophenyltrimethylammonium hapten. The results of the binding plots, using both the Scatchard and Sips analyses, demonstrated that the specifically purified anti-Ap antibodies had a heterogeneity index of 0.6 and contained 2 moles of combining sites per mole protein.

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It is generally accepted that the specificity of an antibody molecule, like that of an enzyme, resides solely in the primary structure of its heavy and light polypeptide chains and is mediated through the effects of primary structure on the conformation of the antigen-combining site (1-3). Therefore, knowledge of the primary structure of the variable regions of a number of antibodies directed against known antigenic determinants is essential to an understanding of the nature of the specificity of the combining sites of antibody molecules as well as to an understanding of the genetic basis of antibody diversity.

The systematic approaches currently being used to produce homogeneous antibody preparations or the production of reasonable quantities of antibodies with markedly restricted heterogeneity include: the purification of antihapten antibodies by immunoadsorption, the use of restricted antigenic stimuli based on simplification of the immunogen (4-13), the screening of human and murine myeloma proteins for antibody-like activity (14-19), and the cloning of plasma cells from specifically immunized animals (20). In some cases, what appear to be homogeneous antibodies have been found by chance (21, 22).

Purification of specific antibodies from a normal antibody population on the basis of chemical, physical, or biological properties has been disappointing to date (23, 24). Antibody preparations obtained by the use of specific immunoadsorbents (23, 25) have invariably failed to meet one or more of the eight criteria which Krause (4) has suggested as evidence of molecular uniformity. Pincus et al. (26), using antibodies directed against type III and type VIII pneumococcal polysaccharides, demonstrated that these antibodies were functionally homogeneous (giving homogeneous Scatchard and Sips plots) but were structurally heterogeneous (giving multiple light and heavy chain electrophoretic species).

Recently, Freedman, Grossberg, and Pressman (27) were able
to fractionate rabbit anti-hapten antibodies into three distinct populations based on differences in contact amino acid residues in the combining sites. Since each of these three populations was still structurally and functionally heterogeneous, other ways of extending this procedure have been tried. The purpose of the present study was to isolate a number of functionally and structurally homogeneous antibodies, directed against a simple well-defined haptenic group, in sufficient quantity to allow structural studies on the combining sites to be performed. Although iso-electric focusing has not allowed the resolution of specific antibodies from whole immunoglobulin preparations, the application of this technique to specifically purified antibodies as described in this paper has permitted the separation of a number of fractions which show evidence of molecular uniformity.

**EXPERIMENTAL PROCEDURE**

**Preparation of Antigen—Azoprotein**—Azoprotein was prepared by coupling bovine γ-globulin with diazotized p-aminophenyltrimethylammonium chloride to give BGG-Ap1 (28). Approximately 20 moles of hapten were coupled per mole of protein.

**Preparation of Antiserum**—The method for preparing and testing rabbit antisera directed against p-azophenyltrimethylammonium was described by Groseberg, Radzinski, and Presman (28).

**Purification and Characterization of Anti-Ap Antibody**—The immunoglobulin fraction of rabbit anti-Ap antisera was prepared by three sodium sulfate precipitations at room temperature (29). Rabbit antisera, pooled from several bleedings, from five individual rabbits (rabbit 7331, allotype a1, a3, b4; rabbit 6031, allotype a1, a3, b4; rabbit 4240, allotype a1, a2, b4; rabbit 2284, allotype a1, a3, b4; rabbit 4058, allotype a1, a3, b4) were the source of anti-Ap antibodies. Anti-Ap 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 immunoadsorbent (30). The adsorbed antibodies were eluted with 1 M propionic acid at 4°C (31). The specifically purified antibody preparations were calculated to contain more than 95% antibody as determined by equilibrium dialysis (32). Purified anti-Ap antibodies from rabbits 2284 and 4240 were kindly supplied by Dr. A. Grossberg (Roswell Park, Buffalo, New York).

**Fractionation of Anti-Ap Antibody Molecules**—Samples of anti-Ap antibodies, from a single rabbit, were fractionated on a preparative scale by liquid isoelectric focusing using the LKB 6101 and 6102 (110-ml and 440-ml) electrofocusing columns (LKB Instruments, Stockholm, Sweden) as described by Vesterberg et al. (33). A stabilizing gradient of 0 to 46% sucrose (Mann, ultrapure), containing carrier ampholytes at a concentration of 1% and covering the range pH 3 to 10 or pH 5 to 8, was used. The sample was applied by mixing it with the carrier ampholyte solution during the loading of the column in the conventional way and electrofocused at a constant voltage of 850 volts for 72 hours at 4°C. However, a modified procedure by which precipitation was often avoided and by which resolution and recovery were improved consisted in running the column for 24 hours without the sample with a plastic tube positioned in the middle of the gradient. Then 2 ml of solution were withdrawn from the column by pumping (LKB 12000 VarioPerpex peristaltic pump). The sample was mixed with the solution and the mixture was pumped back to its original position by means of the same tube. The sample was then electrofocused in the usual manner for an additional 48 to 72 hours at a constant voltage of 850 volts. At the end of the run the contents were pumped slowly from the column and the absorbance at 280 nm was monitored continuously using a Beckman DBG spectrophotometer equipped with a micro flow cell and a 10-inch linear recorder. The pH of the effluent was measured at 4°C using a Radiometer pH meter equipped with a micro combination electrode. The sucrose and carrier ampholytes were removed from the antibody fractions by gel filtration using Sephadex G-50 (fine) in phosphate-buffered saline (0.15 M NaCl, 0.01 M sodium phosphate, 0.02% sodium azide), pH 7.4, at 4°C. The protein was monitored at 280 nm, and the sucrose and carrier ampholytes were monitored at 254 nm using an Isco UA-2 ultraviolet analyzer with a channel alternator (model 580, Specialties Instrumentation Company, Lincoln, Nebraska). Approximately 5 to 20 mg of specifically purified rabbit anti-hapten antibodies were separated on the 110-ml focusing column and 40 to 200 mg of specifically purified rabbit anti-hapten antibodies were separated on the 440-ml focusing column. Carrier ampholytes covering a single pH unit in the 5 to 8 pH range were prepared by focusing 25 ml of 40% w/v carrier ampholytes (pH 5 to 8) in the 110-ml LKB focusing column. The carrier ampholytes covering a single pH unit in the 5 to 8 pH range were prepared by focusing 25 ml of 40% w/v carrier ampholytes (pH 5 to 8) in the 110-ml LKB focusing column. The column was run for 5 days at a constant power of approximately 3 watts. After elution and measurement of pH, the fractions were pooled to give the desired pH range of the carrier ampholyte. The resulting ampholytes were 9% w/v. Blank focusing columns were run at pH 3 to 10, 5 to 8, and 6.2 to 7.4 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 shown in Figs. 1 to 6.

**Determination of Antibody Activity by Equilibrium Dialysis**—The antibody activity of anti-Ap antibody and the focused anti-Ap antibody fractions was determined by equilibrium dialysis with 125I-labeled hapten (125I-labeled p-iodophenyltrimethylammonium) at several different free hapten concentrations, as previously described (32). All binding experiments were carried out in phosphate-buffered saline, pH 7.4, at 4°C. The antibody concentration was 0.33 μg/ml, and the free hapten concentrations at equilibrium ranged from 0.11 μM to 6.2 μM. This concentration range was selected so as to include that concentration of free hapten necessary to half-saturate the antibody. The binding data were analyzed with the use of the Sips equation to obtain values for the average binding constant (Ks), the binding constant (Kd), moles of antibody combining sites per mole of protein (n), and heterogeneity index (a) (34) (see Figs. 8b and 9b). By this method n can be determined to an accuracy of ±5%, providing that the molecular parameters of the protein are known precisely. Figs. 8e and 9e are a plot of the moles of hapten bound per mole of antibody (r) against r/c; c is the concentration of free hapten at equilibrium. This method of plotting was described by Scatchard (35), and first utilized for antibodies by Karush (36). The open symbol (solid square in open square) on the abscissa represents the best value for n. To determine the best value for n, the data was plotted as log

1 The abbreviations used are: BGG-Ap, bovine γ-globulin coupled with diazotized p-aminophenyltrimethylammonium; anti-Ap, rabbit antisera directed against p-azophenyltrimethylammonium; IgG, IgA, and IgM, symbols for immunoglobulins recommended by a conference on human immunoglobulins sponsored by the World Health Organization (38); Fd, symbol for the heavy polypeptide chain component in that fragment of the IgG antibody molecule which contains the antigen-combining site (98); Fe, symbol for the C-terminal half of the heavy polypeptide chain (38).
(r/n) against log e, n was varied, and the value which yields a straight line was selected (34). The binding curves in Figs. 8a and 9a were extended (rather than extrapolated) to the open symbol on the abscissa. The values for $K_a$ and $K_A$ were obtained from the logarthmic plots. The heterogeneity index ($a$) is the slope of the logarthmic plot.

Preparation of $^{131}I$-Labeled Hapten—$^{131}I$-labeled p-iodophenyltrimethylammonium was prepared by isotope exchange with carrier-free $^{131}I$-iodide (28).

Determination of Protein—Protein determinations were usually performed by the modification of the Folin reaction developed by Lowry et al. (37). Otherwise protein concentrations were determined spectrophotometrically assuming an $E_{	ext{it}}^{1}m$ of 14.6 at 280 nm. The molecular weight of antibodies was taken to be 150,000 daltons since the preparations were essentially 100% IgG. No IgA or IgM (38) was detected in the specifically purified antibody samples before isoelectric focusing by either analytical ultracentrifugation or immunoelectrophoresis.

Determination of Amino Acid Compositions—Amino acid analyses were performed on a Beckman 121 automatic amino acid analyzer, equipped with an Infotronics CRS-110A digital integrator, using a 4-hour gradient. The samples were hydrolyzed for 20 hours at 110° in constant boiling HCl (8 n) in ampules sealed after flushing with nitrogen.

Disc Gel Electrophoresis—Anti-Ap antibody and the focused fractions were extensively reduced and alkylated, in 7 M guanidine hydrochloride, using 0.1 M dithiothreitol and 0.22 M iodoacetamide. The reduced and alkylated antibodies were dialyzed against 10 M urea. The urea was freshly prepared and deionized using Rexyn I-300 (39). The p-iodophenylamidine disc electrophoresis was performed at 2.5 mA per tube for 4 hours (39). After electrophoresis the gels were fixed with 5% trichloroacetic acid and 5% sulfosalicylic acid, and then were stained with Coomassie blue for detection of all resolved components (40).

Determination of Allotypic Specificity—The allotypic markers for the light and heavy chains (Fd fragments) of the individual rabbit antibodies were determined qualitatively by Ouchterlony double diffusion analyses (45) and quantitatively by Oudin's method of simple diffusion in gel tubes (46) and by a modified radial diffusion method (47). In each case specific rabbit antisera directed against the appropriate allotypic determinants (a1, a2, a3, b3, b4, b5, and c7) were used. The protein concentrations of the antigens used were 30 mg per ml for the Ouchterlony analyses and 6 mg per ml for the radial diffusion analyses. Antigen concentrations were read from a standard curve and expressed as percentages of the total antigen or protein present. The IgG concentration of the samples tested was determined by radial diffusion analysis with goat anti-rabbit Fc serum.

RESULTS

Separation of Rabbit Anti-Ap Antibodies by Preparative Isoelectric Focusing—The whole immunoglobulin fraction was prepared from five rabbits immunized against BGG-Ap. These preparations were shown to be free of other serum proteins by analytical ultracentrifugation and by either starch gel or acrylamide gel electrophoresis. Typical focusing elution profiles over the pH range 3 to 10 of the immunoglobulin fraction from rabbits 7331 and 6301 are shown in Figs. 1, a and b. These experiments were performed to look at all of the antibodies directed against the Ap hapten in any single antiserum. Almost all of the immunoglobulins focused between pH 5 and 8 on the column. Since the immunoglobulin fraction contained only 10% specific antibodies and since the focusing profiles were so complex, we decided to use antibodies which had been specifically purified on a solid immunoadsorbent.

Typical focusing elution profiles (pH 3 to 10) of specifically purified rabbit anti-Ap antibody are shown in Figs. 2 and 3. Almost all of the antibodies focused between pH 5 and 8. The antibody molecules were distributed between two main fractions with average isoelectric point (pI) values of approximately 5.7 (range 5.0 to 6.0) and 6.3 (range 6.0 to 6.8) for rabbit 7331 (Fig. 2), and approximately 5.8 (range 5.2 to 6.0) and 6.5 (range 6.2 to 7.0) for rabbit 6301 (Fig. 3). There was evidence of further resolution in both main fractions. Since more than 95% of the
anti-Ap antibodies had isoelectric points between pH 5 and 8, this pH range was used throughout the column in an attempt to get better fractionation.

Two typical focusing elution profiles (pH 5 to 8) of specifically purified anti-Ap antibody from rabbits 7331 and 6301 are shown in Figs. 4 and 5. The two differ in detail, but show overall similarities. Both divided into the two major fractions seen in Figs. 2 and 3, but are shown resolved into a number of discrete fractions. The profiles of the two preparations differed in the number and pI values of their individual antibody components. The focusing elution profiles in Figs. 4 and 5 show that specifically purified antibodies could be further separated into as many as 15 to 20 peaks (including 4 to 8 major, well resolved peaks).

To establish that these fractions were not being generated during the focusing procedure, focused Fraction B (Fig. 4) was focused over the pH range 5 to 8. Focused Fraction B has a pI value between pH 6.8 and 7.0. The refocused Fraction B elution profile is illustrated in Fig. 6a. The refocused antibody fraction gave only one symmetrical peak with a pI value between pH 6.8 and 7.0.

Evidence for Structural Homogeneity in Focused Antibody Fractions—The homogeneity of three of the focused fractions (Fractions...
Fig. 6. a, the liquid isoelectric refocusing elution profile of 7 mg of focused Fraction B anti-Ap antibody (Fig. 4) in the pH 5 to 8 gradient range at 850 volts for 72 hours, using the 110-ml electrofocusing column. The — — — — , same as in Fig. 1a. b (inset), the liquid isoelectric refocusing profile of 5 mg of focused Fraction B anti-Ap antibody (a) in the pH 6.2 to 7.4 gradient range at 850 volts for 72 hours, using the 110-ml electrofocusing column. — — — — , same as in Fig. 1a.

The fully reduced and alkylated purified anti-Ap antibodies and several focused fractions were subjected to alkaline disc gel electrophoresis in 10 M urea. Photographs and densitometer tracings from typical gel patterns of focused fractions from two individual rabbit preparations are shown in Fig. 7, a and b. In each case, the light chain pattern of the whole anti-Ap antibody is shown. This is polydisperse and shows several distinct zones characteristic of a mixture of immunoglobulins (48, 49). The gels containing the light chains from the focused antibody fractions showed patterns which were much less disperse, and in some cases showed only a single major light chain band (Fig. 7, a and b, Fractions A' and B). It is evident from the elution profiles shown in Figs. 4 and 5 that Fractions A' and B separate more completely from neighboring fractions, and their gel patterns are closer to the single light chain ideal. Fraction D' comes from a more complex region of the focusing elution profile (Fig. 5).

Fig. 7. a, densitometric tracings and photographs of the alkaline urea disc gel electrophoretic patterns of fully reduced and alkylated specifically purified anti-Ap antibody (rabbit 7331) and focused Fractions (Fr.) B (Fig. 4). The densitometric tracings were taken, using a Gelman Gelscan apparatus with a linear recorder, from a 3-cm portion of the gel in which the light chains were located. I.E.F., isoelectric focusing fractions. b, densitometric tracings and photographs of the alkaline urea disc gel electrophoretic patterns of fully reduced and alkylated specifically purified anti-Ap antibody (rabbit 6301) and focused Fractions A' and D' (Fig. 5). Conditions the same as in a.
and its light chain pattern, which is typical of these fractions, is more disperse (Fig. 7b).

The distribution of the light and heavy chain allotypic markers on the focused fractions and the specifically purified antibody from rabbit 6301 was tested by Dr. S. Dubiski (University of Toronto, Toronto, Canada), and Dr. R. Mage (National Institutes of Health, Bethesda, Maryland). All fractions identified in Fig. 5 were tested together with several unidentified minor fractions (I, II, III) from the same run. All were tested against antisera specific for a1, a2, a3, b4, b5, and c7. Rabbit 6301 showed b4 and c7 markers for κ and λ light chains, respectively. The b5 marker was absent. The rabbit was heterozygous with respect to the heavy chain (Fd) markers reacting with both anti-a1 and anti-a3 antisera.

On Ouchterlony double diffusion analysis, the b4 and a1 markers were present in all fractions in roughly equal concentration. However, Fractions A', B', and C' gave negative or only faintly positive reactions with anti-a3 antisera, while Fractions D', E', F, I, II, and III reacted strongly with anti-a3 antisera. On the basis of Oudin analysis of the a3 content of the anti-Ap antibody, the concentration used in the Ouchterlony test (30 ng per ml) was 10 times the concentration needed to produce a strongly positive precipitin line.

These observations were quantitated by radial immunodiffusion analysis. The results are presented in Table I together with quantitative data for the total immunoglobulin fraction from this rabbit. The a1 marker was present on approximately 40% of the heavy chains of the total IgG fraction and the a3 on the remainder. Of the light chains, 9% contained the c7 marker, and the remainder of the light chains, the b4 allotypic marker. These ratios were altered in the specifically purified antibody which contained 90% a1 and 10% a3 on the heavy chains. The c7 marker could no longer be detected (<1%), and only the b4 marker, characteristic of the κ light chain, was found. The further fractionation of the a3 allotypic marker as a result of isoelectric focusing is seen in Fractions A', B', and C'. The relative concentration of a1 in Fractions I, II, III, D', and E' was 88 to 89%, slightly reduced from that of the specifically purified antibody, while the relative concentration of a1 in Fractions A', B', and C' was increased to 95 to 96% (Table I).

Fractions A' and B' (Fig. 5) were refocused over a pH range of 0.5 units on either side of their respective isoelectric points. The fully reduced and alkylated light chains of these preparations and of whole anti-Ap antibody were isolated and subjected to automatic sequence analysis by Dr. W. Terry (National Institutes of Health, Bethesda, Maryland). The amino acids were identified and quantitated by both gas chromatography and amino acid analysis. Preliminary results from the sequence analysis of the light chains from anti-Ap antibody show significant amounts of both alanine and aspartic acid at the N-terminal position. The next seven residues show an even greater degree of heterogeneity and the composition of the sequence closely resembles those already published for light chains from normal rabbit pools (12, 60). Light chains prepared from the re-focused fractions gave only a single N-terminal amino acid, in each case alanine, and to date the sequences (21 residues of Fraction A' and 11 residues of Fraction B') show a degree of homogeneity similar to that seen in Bence-Jones proteins.3

**Evidence for Functional Homogeneity in Focused Antibody Fractions**—Binding affinity may be used as an indicator of functional homogeneity in antibody molecules. The results of equilibrium dialysis for the specifically purified whole anti-Ap antibody (rabbit 7331) and four of the major focused antibody fractions (Fig. 4) with the 125I-labeled p-nitrophenylmethyllummonium hapten are plotted in Figs. 8a and 9a by the Scatchard analysis according to the equation \( r/c = K_n - K_r \). The data is also plotted by the logarithmic form of the Sips distribution function according to the equation \( \log (r/n - r) = a \log c + a \log K_A \) (Figs. 8b and 9b). The equilibrium dialysis results (Table II) show that all of the antibody fractions had 2.0 ± 0.1 moles of combining sites per mole of protein (assuming a molecular weight of 150,000 daltons). The unfraccionated anti-Ap antibody had a heterogeneity index (a) of 0.6 ± 0.03 (Fig. 8b), while the focused anti-Ap antibody fractions all had heterogeneity indices of 1.0 ± 0.05 (based on the slopes of the logarithmic plots, Figs. 8b and 9b). The average association constant (\( K_A \)) for the purified anti-Ap antibody was 4.0 × 10^6 M^-1 (at 4°C) while the association constant (\( K_A \)) values for the focused anti-Ap antibody fractions ranged from 3.0 to 4.4 × 10^5 M^-1 (Table II). These findings indicate that the separated anti-Ap antibody fractions are monodisperse with respect to binding affinity.

**Comparison of Amino Acid Composition of Anti-Ap Antibody (Rabbit 7331) and Isoelectrically Focused Fractions**—A comparison of the over-all amino acid composition of rabbit 7331 anti-Ap antibody and four of the major focused fractions (Fig. 4) is shown in Table III. Although there are no great differences in the amino acid distribution among the focused anti-Ap antibody fractions, significant differences were found in the arginyl and

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3 W. D. Terry and M. H. Freedman, unpublished results, papers in preparation.
isoleucyl residues and to a lesser extent in the lysyl, alanyl, valyl, anderyl residues. A comparison of the over-all charge content of the focused fractions \( ([\text{Asp} + \text{Glu}] - ([\text{Lys} + \text{Arg}]) \) shows that focused Fraction A, the least acidic, lies closest to the cathode with a pI value of 7.2, and focused Fraction D, the most acidic, lies closest to the anode with a pI value of 5.8. The focused Fractions B and C have charge values intermediate between Fractions A and D, and they have pI values of 7.0 and 6.4. This effect is seemingly caused more by changes in the lysyl and arginyl residues than by changes in the dicarboxylic acids.

Although the ammonia determinations are not corrected for background or destruction of amino acids, some comparison may be made between the runs which were consecutive on the same column of the Beckman 121 autoanalyzer. The fact that there was no consistent trend in the distribution of ammonia across the range of isoelectric points (Table III) suggests that the separation of the focused antibody fractions is not solely due to differences in amide content.

**Recovery of Anti-Ap Antibody Fractions Following Isoelectric Focusing**—The over-all recoveries and yield of individual frac-

![Fig. 8](image1.png)

**Fig. 8.** a, binding of \( ^{125}I \)-labeled \( p \)-iodophenyltrimethylammonium hapten by purified rabbit 7331 anti-Ap antibody and isoelectric focused Fraction (I.E.F.) B (Fig. 4), measured by equilibrium dialysis \( (4^*) \); \( r \), moles of hapten bound per mole of protein (molecular weight assumed to be 150,000 daltons); \( c \), the free hapten concentration; \( n \), the moles of binding site per mole protein. Each point represents the mean of triplicate determinations with an average deviation from the mean of \( \pm 0.9\% \). The \( n \) value (abscissa, solid square in open square) is the value obtained from the theoretical curve which best fits the data by application of the Sips equation in its logarithmic form \( (\text{see } b) \). b (inset), the same data plotted according to the logarithmic Sips equation to evaluate homogeneity \( (n = 2.0) \).

![Fig. 9](image2.png)

**Fig. 9.** a, binding of \( ^{125}I \)-p-iodophenyltrimethylammonium hapten by isoelectric focused fractions (I.E.F.) A, C, and D (Fig. 4), measured by equilibrium dialysis \( (4^*) \). Conditions the same as in Fig. 8a. b (inset), the same data plotted according to the logarithmic Sips equation to evaluate homogeneity \( (n = 2.0) \).

**TABLE II**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Combining sites</th>
<th>( K_a \times 10^{-3} )</th>
<th>Heterogeneity index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Specifically purified anti-Ap antibody (control)</td>
<td>2.0</td>
<td>4.0*</td>
<td>0.6 ± 0.03</td>
</tr>
<tr>
<td>Anti-Ap antibody (Fraction A)</td>
<td>2.0</td>
<td>3.0</td>
<td>1.0 ± 0.05</td>
</tr>
<tr>
<td>Anti-Ap antibody (Fraction B)</td>
<td>2.0</td>
<td>3.5</td>
<td>1.0 ± 0.05</td>
</tr>
<tr>
<td>Anti-Ap antibody (Fraction C)</td>
<td>2.0</td>
<td>3.7</td>
<td>1.0 ± 0.05</td>
</tr>
<tr>
<td>Anti-Ap antibody (Fraction D)</td>
<td>2.0</td>
<td>4.4</td>
<td>1.0 ± 0.05</td>
</tr>
</tbody>
</table>

* The binding constant from the logarithmic form of the Sips equation.

* The number of moles of combining sites per mole protein \( (n) \) was obtained from the theoretical curve which fitted the data by application of the Sips equation in its logarithmic form \( (\text{see } "\text{Experimental Procedure}"\)\). These values have an estimated reliability of \( \pm 5\% \).

* \( K_a \) value, average binding constant.

* Focused antibody fractions (see Fig. 4).
TABLE III

Amino acid compositions of anti-Ap antibody (rabbit 7331) and the electrofocused fractions (Fig. 4)

The values reported for amino acid compositions are residues recovered per molecule of antibody expressed as the nearest integer normalized to a value of 90 for leucine. Values for threonine and serine are not corrected for losses during hydrolysis.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Anti-Ap antibody</th>
<th>Focused</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Samples</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Fraction A (pI = 6.2)</td>
<td>Fraction B (pI = 6.0)</td>
</tr>
<tr>
<td>Lysine</td>
<td>69</td>
<td>70</td>
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<td>Histidine</td>
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<tr>
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<td>Threonine</td>
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</tr>
<tr>
<td>Glutamic acid</td>
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<tr>
<td>Leucine</td>
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<td>(90)</td>
</tr>
<tr>
<td>Tyrosine</td>
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<td>55</td>
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<tr>
<td>Phenylalanine</td>
<td>44</td>
<td>46</td>
</tr>
</tbody>
</table>

* The values represent the mean of triplicate amino acid analyses with an average deviation from the mean of ±3%.

TABLE IV

Recovery of specifically purified anti-Ap antibody fractions following fractionation by isoelectric focusing and Sephadex G-50 gel filtration

<table>
<thead>
<tr>
<th>Sample</th>
<th>Weight of antibody fraction (mg)</th>
<th>% of total antibody population</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fraction A*</td>
<td>4.1</td>
<td>6.0</td>
</tr>
<tr>
<td>Fraction B*</td>
<td>7.0</td>
<td>10.3</td>
</tr>
<tr>
<td>Fraction C*</td>
<td>4.4</td>
<td>6.5</td>
</tr>
<tr>
<td>Fraction D*</td>
<td>6.0</td>
<td>8.8</td>
</tr>
<tr>
<td>Fraction A**</td>
<td>4.3</td>
<td>6.3</td>
</tr>
<tr>
<td>Fraction B**</td>
<td>7.8</td>
<td>11.5</td>
</tr>
<tr>
<td>Fraction C**</td>
<td>4.6</td>
<td>6.8</td>
</tr>
<tr>
<td>Fraction D**</td>
<td>5.4</td>
<td>7.9</td>
</tr>
<tr>
<td>Fraction E**</td>
<td>3.6</td>
<td>5.3</td>
</tr>
</tbody>
</table>

* From rabbit 7331 (Fig. 4), 68 mg of specifically purified anti-Ap antibody was applied to the column. Total recovery of antibody following elution from the focusing column and gel filtration on Sephadex G-50 was 44 mg.

** From rabbit 6301 (Fig. 5), 68 mg of specifically purified anti-Ap antibody was applied to the column. Total recovery of antibody following elution from the focusing and gel filtration on Sephadex G-50 was 49 mg.

It is well known that the immune response is generally characterized by the production of antibodies which are heterogeneous with respect to their structural and functional properties. In contrast, myeloma proteins have been shown to be structurally homogeneous and, in a few instances, to be functionally homogeneous (14-19). There are several advantages in working with classically induced normal antibodies as compared with the myeloma proteins demonstrating antibody-like activity. The main advantage is that the antigen, against which the combining site of the antibody is directed may be chosen and thus an immunogen and antibody system can be selected to meet the investigator's purpose. Consequently, it is desirable to develop a method for the purification of homogeneous populations of antibody molecules which is independent of the nature of the immunogen and of the animal used to prepare the antibodies.

In the past, fractionation of heterogeneous antibody populations based on the physical, chemical, or antigen binding properties of the antibodies has been unsuccessful, since the fractionated antibodies were often as heterogeneous as the unfractionated antibodies (23, 24). Using liquid isoelectric focusing, we have been able to separate, on a preparative scale, specifically purified rabbit antibodies directed against the p-azophenyltrimethylammonium hapten into a number of discrete fractions.

Criteria for homogeneity of antibodies have been classified under three headings: charge, functional, and structural. Although each is independently insufficient as evidence for homogeneity, a number taken together provide a more convincing case. The finding that two or three of the focused anti-Ap antibody fractions fulfill to a greater or lesser degree all of these independent criteria is a good indication that isoelectric focusing can be used to produce, from a single rabbit, several antibody fractions sufficiently homogeneous for structural analysis. The sequences of two or more antibodies from the same animal directed against a single hapten will be very useful in determining the basis of antibody diversity. Specifically purified antibody is itself unsuitable for structural studies and shows all of the properties characteristic of heterogeneous proteins.

The focused antibody fractions were shown to be monodisperse with respect to charge by a variety of analytical electrophoretic procedures including cellulose acetate, starch gel, isoelectric focusing in acrylamide gel, and isoelectric focusing in a pH gradient extending only 0.6 pH units on either side of the pI value (Fig. 6c). In contrast, the parent unfractionated antibody demonstrated polydispersity in all types of electrophoresis and separated into more than 20 fractions in isoelectric focusing between pH 5 and 8 (Figs. 4 and 5).

The functional homogeneity of the focused antibody fractions was demonstrated by hapten binding studies. All of the focused antibody fractions tested had 2 moles of combining sites per mole of protein and a heterogeneity index of 1.0 ± 0.05 (Figs. 8 and 9, Table II). The specifically purified antibody also had 2 moles of combining sites per mole of protein, but had a heterogeneity index of 0.6 ± 0.03 (Fig. 8 and Table II) typical for heterogeneous antibody preparations.

The structural homogeneity of the light and heavy polypep-
tide chains of the focused antibody fractions was suggested by
disc electrophoresis, sequence analysis, and determination of
the distribution of allotypes. The focused fractions showed
monodisperse light chains on alkaline urea disc electrophoresis,
whereas the specifically purified antibody showed 6 to 8 light
chain components (Fig. 7, a and b). The presence of a single
zone after alkaline urea electrophoresis of immunoglobulin light
chains has only been seen with myeloma proteins or antibody
preparations which have been shown to be monodisperse on
sequence analysis (4, 13).

The light chains of the focused antibody Fractions A' and B'
gave a single N- terminal amino acid and have continued to show
a coherent singular sequence as far as they have been sequenced
(21 and 11 residues, respectively). In their degree of homo-
genicity these sequences resemble those of Bence-Jones proteins
sequenced in the same instrument. In contrast, the unfrac-
tioned, specifically purified antibody gave 2 major residues in
the N-terminal position and continued to display great sequence
heterogeneity for the next 7 residues examined. This sequence
resembled those found for a pooled immunoglobulin fraction
from a number of rabbits (19, 50).

The b4 light chain allotype was present in all fractions and in
the specifically purified antibody. No b5 or c7 light chain all-
types were detected in either the focused fractions or the specifi-
cally purified antibody, indicating that the light chains were of
one allele and solely of the A class. The 9% of A light chains
observed in the whole immunoglobulin fraction was excluded
during the purification procedure.

The homogeneity of the heavy chains was tested by an exami-
nation of the distribution of the a1, a3 allotypic markers in the
focused fractions. The heavy chains from the whole immuno-
genfracin contained both a1 and a3 allotypic markers,
indicating that the rabbit was heterozygous (a1, a3); however,
the concentration of the a1 allotypic marker increased during the
purification from 40% in the whole immunoglobulin to 90% in
the focused fraction (Table I). Kindt et al. (31) reported a resi-
dual 8% of the excluded (a3) allotype in purified antibodies,
directed against a streptococcal carbohydrate antigen, which
was subsequently shown to be suitable for sequence analysis.
In our case, part of this purification was achieved during the
separation of the specifically purified antibody but the final con-
centration from 90% to 96% was produced by isoelectric focus-
ing. The selective exclusion of the a1 allotype from specifically
purified antihapten antibodies has already been noted by Lark,
Eisen, and Dray (32). They found that antibodies directed
against the 2,4-dinitrophenyl or p-arsanilate haptons produced in
heterozygous rabbits were enriched in a3 or b4 allotypic
markers. Thus, two or three antibody fractions, directed against
known haptenic determinants and derived from a single rabbit,
have been prepared which satisfy a number of mutually
independent criteria for homogeneity. The most definitive test
of homogeneity is the amino acid sequence and experiments are in
progress to determine as much of the NH2-terminal variable se-
quence as possible. Amino acid analysis gives an indication of differences in the
composition of the isolated antibody fractions. Comparative
analyses shown in Table III suggest that minor differences do
occur between the separated fractions. Over-all differences in
the number and charge of the ionizable groups are consistent with
their relative isoelectric points. The relative distribution of ammonia in the amino acid analyses was not consistent with
the idea that the separation is based solely on differences in
amide content.

We were unable to separate successfully the antihapten anti-
body populations present in the immunoglobulin fraction pre-
bred by salt precipitation of the immune sera since the normal
immunoglobulins interfered with the resolution of the specific
antibodies on isoelectric focusing (Fig. 1, a and b). The electro-
focusing of the rabbit immunoglobulin component resembles
previously reported focusing separations of human Cohn Fraction
II (65).

The focusing procedure for fractionating specifically purified
antibodies was not restricted to the purification of rabbit anti-Ap
antibodies. Using the same procedure, we have been able to
fractionate individual rabbit and goat specifically purified anti-
bodies directed against a number of positively charged, nega-
tively charged, and neutral haptons.2

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