Partial Separation on DEAE-Sephadex of Antibodies Reactive with Plant Viruses and their Protein Subunits

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SUMMARY

Antibodies induced by the injection of alfalfa mosaic virus (AMV) or the protein of turnip crinkle virus (TCV) reacted with the viruses and their respective protein subunits in gel-diffusion tests. The ratios of virus-reactive antibodies to protein-reactive antibodies were different in two fractions obtained by chromatography of the sera on DEAE-Sephadex. The first DEAE-Sephadex fraction contained a greater proportion of virus-reactive antibodies than did the second fraction or the unfractionated antiserum. Electrophoretic mobility measurements at pH 8.6 showed that AMV and TCV had a greater negative charge per unit surface area than did their respective proteins.

INTRODUCTION

Antibodies of the immunoglobulin G class from different rabbit antisera were separated into two types by chromatography on DEAE-Sephadex A-50 medium (Sela, Givol & Mozes, 1963; Sela & Mozes, 1966; Mozes, Robbins & Sela, 1967; Rude, Mozes & Sela, 1968). The induction of antibodies of these types is a function of the net electrical charge on the antigen molecule at physiological pH, rather than immunization technique or the animal. Antibodies to antigens carrying a large net negative charge (acidic) were found predominantly in the first chromatographic fraction (fraction B) eluted from a DEAE-Sephadex column, whereas antibodies to antigens carrying a large net positive charge (basic) were mostly found in the second fraction (fraction C). Antigens carrying small net negative or positive charges at physiological pH values did not induce a predominance of antibodies of either type. Fraction C antibodies have a greater negative electrophoretic mobility than fraction B antibodies. Hence highly basic antigens induce the formation of more acidic antibodies and highly acidic antigens induce a predominance of less acidic antibodies.

Allen & Tremaine (1965) found two groups of antibodies in antisera against Prunus necrotic ringspot virus: group A reacted with both virus particle and subunit protein antigens and group B was specific for virus particles. Only group A antibodies were induced by injection of the subunit protein. These antibodies of differential specificity were partially separated by agar gel electrophoresis. The majority of the antibodies specific for the Prunus necrotic ringspot virus particle had a lower negative electrophoretic mobility (less acidic) than the antibodies which reacted with the protein subunit. Electrophoretic studies of the antigens demonstrated that the virus particles had a greater negative mobility than the protein subunit. If groups A and B antibodies are induced by protein subunit and virus

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particle, respectively, the separation of antibody specificities is explicable in terms of the charge density on the inducing antigens.

Although the particles of most plant viruses have a negative electrophoretic mobility at pH 7.0, the electrophoretic mobilities of their protein subunits are seldom reported. The mobilities of the protein subunits from tobacco mosaic virus and bromegrass mosaic virus have been determined; both are considerably less than the mobilities of their respective virus particles (Hamilton, 1961; Ansevin, Stevens & Lauffer, 1964). The purpose of the present study was to compare the mobilities of the virus particles and protein subunits of two viruses, turnip crinkle virus (TCV) and alfalfa mosaic virus (AMV); to prepare antisera containing virus particle-reactive and protein-reactive antibodies; to obtain fraction B and fraction C antibodies by chromatography of the antisera on DEAE-Sephadex; and to ascertain if virus particle-reactive antibodies and protein-reactive antibodies are predominantly of fraction B type and fraction C type, respectively.

METHODS

Virus and protein preparation. A culture of turnip crinkle virus (TCV) was obtained from Dr R. Haselkorn, University of Chicago. A culture was established by selecting a local lesion from Chenopodium amaranticolor Coste and Reyn and the virus was propagated in sap-inoculated Brassica pekinensis Rupr plants. About four weeks after inoculation the infected plants were harvested and ground in a meat grinder. The sap, expressed from the pulp by squeezing through cheesecloth, was adjusted to pH 5.0 with 1 N-acetic acid and left overnight at 4 °C. After low speed sedimentation, the virus was pelleted in a no. 30 rotor in the Spinco Model L centrifuge at 28000 rev/min for 90 min. The pellets were dissolved in 0.02 M-tris-HCl at pH 7.0 and given another cycle of sedimentation and a density gradient sedimentation. Examination of purified preparations in the analytical ultracentrifuge showed the presence of a single component with a s20,w of 126S.

TCV protein (TCV-P) was prepared by a modification of Leberman’s (1968) method. The virus preparation at 20 mg/ml was mixed with an equal vol. of 0.1 M-tris-HCl buffer, pH 7.0, containing 2 M-KCl and stored at 4 °C for 4 days. This partially degraded virus preparation was chromatographed on a 100 x 2.5 cm column of Sephadex G-200 in 0.1 M-tris-HCl, pH 7.0, containing 0.5 M-KCl, at 20 ml/h and 4 °C. The undegraded virus and RNA were eluted in the void vol. and protein contaminated with less than 0.5% RNA was eluted after. The protein was not stable and gradually precipitated on storage. However, it was stable after dialysis against 0.05 M-veronal buffer, pH 8.6. Degradation of the virus in 0.1 M-tris-HCl buffer, pH 9.0, containing 0.5 M-KCl (Leberman, 1968) greatly reduced the yield of protein and some precipitated protein was eluted in the void vol. with the RNA.

A potato strain of alfalfa mosaic virus (AMV) was purified as described previously (Tremaine & Stace-Smith, 1969). To ensure absence of host material, only the bottom component was removed from density-gradient columns. The protein of AMV was isolated by the method of Kelly & Kaesberg (1962) omitting sodium dodecyl sulphate. The protein remained soluble for a few days at 4 °C but was stable for at least one week after dialysis against 0.05 M-veronal, pH 8.6.

Concentrations of virus particle and protein preparations were determined from E280 and E280, respectively. Extinction coefficients of 6 and 0.6 cm²/mg were used for virus particles and protein, respectively.

Serology. All antisera were produced in rabbits by an intramuscular thigh injection of 1 mg of antigen in Freund’s complete adjuvant followed by two intravenous injections of
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1 mg of antigen at one-week intervals. The animals were bled once a week for approx. 10 weeks and given an intravenous injection of 1 mg of antigen after each bleeding. Injection of larger quantities of antigen yielded antisera of much lower titres.

Gel-diffusion tests were conducted in triplicate in square plastic Petri dishes, 9 x 9 cm, containing 12.5 ml of agar. One-twentieth millilitre portions of antigens and antisera were placed in wells of 6 mm diam. The gel was prepared at a concentration of 0.7% Ionagar no. 2 in 0.015 M-sodium azide, 0.02 M-tris-HCl, pH 7.4, and containing 0.5 M or 0.2 M-NaCl. Undegraded virus particles (1 mg/ml) were used as antigens. TCV and AMV partially degraded in the 0.5 M- and 0.2 M-NaCl media, respectively, and each reacted to form two precipitin bands: undegraded virus particles reacted with antibodies close to the antigen well and protein reacted with antibodies closer to the antiserum well. The titre of antibodies reactive with TCV protein was low in sera from rabbits injected with the virus; however, sera reactive with virus and with greater titres of protein-reactive antibodies were obtained by the injection of TCV protein. Sera having satisfactory titres of AMV protein-reactive antibodies were obtained by injection of intact virus particles.

Electrophoresis. Antigens and antibodies were electrophoresed at 4 °C in a Gelman electrophoresis chamber on 2.5 x 15 cm strips of cellulose acetate in 0.05 M-veronal buffer, pH 8.6. The field strength was 10 V/cm and antigens and antibodies were electrophoresed for 2 h and 6 h, respectively. The strips were stained with saturated nigrosine in 2% acetic acid and destained by rinsing in 2% acetic acid. The electropherograms were cleared in paraffin oil and scanned using a Joyce-Loebl microdensitometer.

Chromatography. The sera (10 ml) were chromatographed at 4 °C on a 2.5 x 22 cm column of DEAE-Sephadex A-50 medium, after dialysis for at least 24 h against 0.02 M-potassium phosphate buffer, pH 8.0. The flow rate was 20 ml/h and 8 ml of eluant was collected in each test tube. After passage of 100 ml of this buffer through the column the first fraction (fraction B) of immunoglobulin G (Ig G) was eluted. A gradient of increasing molarity was then applied by connecting a 600 ml beaker containing 500 ml of 0.3 M-potassium phosphate buffer pH 8 with a 1000 ml beaker containing 750 ml of the starting buffer. After elution with 120 ml of this gradient the second fraction (fraction C) of Ig G was eluted. The contents of the tubes containing fractions B or C were pooled, concentrated by (NH₄)₂SO₄ precipitation and dialysed against 0.15 M-NaCl. The vol. of each pooled fraction was adjusted to 5 ml.

Sela & Mozes (1966) used a 2.3 x 33 cm column of DEAE-Sephadex A-50 in their separation of fractions B and C. We found the use of comparable vol. in our study (2.5 x 27 cm columns) to be impractical because the fractions were eluted too slowly. With some batches of DEAE-Sephadex A-50, an increase in the molarity of the starting buffer to 0.025 M or higher was required for good separations (M. Sela, personal communication).

RESULTS

Electrophoresis of AMV, TCV and their proteins

5 µl of preparations of AMV, AMV protein, TCV and TCV protein were spotted onto cellulose acetate strips and electrophoresed for 2 h at 10 V/cm. Under these conditions AMV and TCV moved 32 and 10 mm, respectively, from their point of application to the anode (Fig. 1 a, b). Although TCV moved as a discrete spot, a considerable amount of tailing was observed following the heavily stained AMV spot, probably caused by the degradation of the virus during electrophoresis. The AMV protein moved 2 mm toward the anode and the TCV protein moved 1.5 mm toward the cathode (Fig. 1 c, d). The slight
Chromatography of antisera on DEAE-Sephadex

The separations obtained with TCV protein antiserum and AMV antiserum on DEAE-Sephadex are shown in Fig. 2(a) and Fig. 2(b), respectively. Serum components in TCV protein antiserum (Fig. 2a) fractionated into four peaks. Sela & Mozes (1966) obtained similar patterns with rabbit antiserum and identified components in fractions comparable to those in fractions 12, 26 and 30 as three types of Ig G which yield papain fragments II + III, I + II + III and I + III, respectively. Albumin, macroglobulins and other serum components are eluted after the third Ig G peak (fraction 35 to end). The contents of fractions 8 to 17 and of fractions 24 to 34 were termed fractions B and C respectively. The protein content of fraction C was approx. three times that of fraction B.

Serum components in AMV antiserum (Fig. 2b) were not as well resolved as those in TCV protein antiserum. The protein content of fraction C (fractions 23 to 35) was approx. five times that of fraction B (fractions 5 to 20).

Fractions B and C of the TCV protein antiserum (Fig. 1e, f) moved 19 and 14 mm, respectively, toward the cathode upon electrophoresis at pH 8.6 for 6 h at 10 V/cm. In an experiment not shown here fractions B and C from the AMV antiserum moved 20 and 14.5 mm towards the cathode under the same electrophoretic conditions. Although Ig G movement of TCV protein to the cathode was the result of electroendosmotic flow in this direction and does not indicate a net positive charge on the TCV protein.

Fig. 1. Microdensitometer patterns of cleared electropherograms on cellulose acetate stained with nigrosine. The origins were marked with a pencil. (a) alfalfa mosaic virus particles; (b) turnip crinkle virus particles; (c) alfalfa mosaic virus protein; (d) turnip crinkle virus protein; (e) and (f) fractions B and C, respectively, from chromatography of turnip crinkle virus protein antiserum on DEAE-Sephadex. Electrophoresis was for 2 h at 10 V/cm for Fig. 1(a) to (d), and for 6 h for Fig. 1(e) and (f).

Fig. 2. The chromatography of 10 ml of rabbit antiserum on DEAE-Sephadex at 4 °C. Elution was started with 180 ml of 0.02 M-potassium phosphate buffer, pH 8, then a gradient of 780 ml of this buffer and 500 ml of 0.5 M-potassium phosphate buffer, pH 8 was applied. The flow rate was 20 ml/h and fractions are 8 ml. (a) TCV protein antiserum. (b) AMV particle antiserum.
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Fig. 3. The reaction of fractions B and C of turnip crinkle virus protein antiserum with virus particle and protein antigens in gel diffusion tests. Fractions B and C in twofold serial dilution were placed in the wells in rows 1 and 4 and rows 3 and 6, respectively. Undegraded virus was placed in the wells in rows 2 and 5. The dilution end points of virus particle-reactive antibodies are 512 and 128 for fractions B and C, respectively. The dilution end points of protein-reactive antibodies are 4 and 16 for fractions B and C, respectively.

Table 1. Dilution end points of antisera and DEAE-Sephadex fractionated antisera determined by titration against virus preparations in gel-diffusion tests

<table>
<thead>
<tr>
<th>Precipitin zone</th>
<th>Anti TCV unfract.-tionated</th>
<th>Anti TCV B*</th>
<th>Anti TCV C*</th>
<th>Anti AMV unfract.-tionated†</th>
<th>Anti AMV B†</th>
<th>Anti AMV C†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Virus particle (V)</td>
<td>1024</td>
<td>512</td>
<td>128</td>
<td>128</td>
<td>64</td>
<td>64</td>
</tr>
<tr>
<td>Protein (P)</td>
<td>16</td>
<td>4</td>
<td>16</td>
<td>16</td>
<td>0</td>
<td>16</td>
</tr>
<tr>
<td>Ratio V/P</td>
<td>64</td>
<td>128</td>
<td>8</td>
<td>8</td>
<td>∞</td>
<td>4</td>
</tr>
</tbody>
</table>

* Gel diffusion medium contained 0.5 M-NaCl.
† Gel diffusion medium contained 0.2 M-NaCl.

molecules are negatively charged at pH 8.6, electroendosmotic flow causes them to move to the cathode. On the basis of similar results Sela & Mozes (1966) concluded that Ig G of fraction B had a smaller net negative charge than Ig G of fraction C.

Serological tests

Gel-diffusion tests were done with 0.05 ml portions of twofold serial dilutions of fractions B and C and samples of unfractionated antisera. Samples of 0.05 ml of undegraded TCV at 1 mg/ml were placed in a row of 5 wells and various dilutions of the antiserum fractions were placed in two rows of six wells above and below the antigen wells (Fig. 3). TCV diffused into the agar containing 0.5 M-NaCl and some virus particles dissociated into subunit protein; the virus particle and protein reacted to form precipitin lines close to the antigen well and close to the antiserum well, respectively. The same method was used with AMV antigens and antiserum fractions, except that the agar gel contained 0.2 M-NaCl.

The concentrations of virus particle-reactive and protein-reactive antibodies in sera and
fractions were determined by the dilution end point, i.e. the reciprocal of the greatest dilution of the antiserum producing a visible reaction. A titration of fraction B and C antibodies from TCV protein antiserum (Fig. 3) showed virus particle-reactive antibody titres of 512 and 128 for fraction B and C, respectively, and protein-reactive antibody titres of 4 and 16 for fraction B and C, respectively.

The results of titration experiments with fractionated and whole TCV protein antisera, and with fractionated and whole AMV antisera are summarized in Table I. Identical results were obtained in three experiments with freshly prepared antiserum dilutions. The ratio of virus particle-reactive to protein-reactive antibodies from both antisera was increased in fraction B and decreased in fraction C.

Comparison of the actual quantities of virus particle- or protein-reactive antibodies in the fractions is difficult. Sela & Mozes (1966) used the quantitative precipitin technique to determine the weight of specific antibodies in each of the fractions. The use of this technique with an antigen in an equilibrium between dissociated and associated states was not attempted. The titres of virus particle- and protein-reactive antibodies are a fair indication of the antibody content since each fraction was adjusted to one-half the original vol. of unfractionated serum. The titre of TCV particle specific antibodies in fraction B was fourfold greater than that of fraction C, whereas the titre of TCV protein-reactive antibodies in fraction C was fourfold greater than that of fraction B. Upon fractionation of AMV antiserum, all the detectable protein-reactive antibodies were found in fraction C. The titres of virus particle-reactive antibodies in fractions B and C from the AMV antiserum were identical. However, since the Ig G content of fraction B was one-fifth that of fraction C, the ratio of virus particle-reactive antibodies to total Ig G protein was greater in fraction B than fraction C.

DISCUSSION

Antisera from rabbits injected with AMV or TCV-protein reacted with appropriate virus preparations in gel-diffusion tests, in each case showing two lines of precipitation. As the rate of diffusion of the virus particle is much slower than that of the subunit protein, it was assumed that the line nearest to the antigen well was formed from virus particle-reactive antibodies, and the line nearest the antibody well from protein-reactive antibodies. In this way it was possible to estimate the proportion of virus particle-reactive to subunit-reactive antibodies in rabbit antisera. The formation of both anti-virus particle and anti-protein antibodies in the same animal is convenient because it eliminated variations in the relative levels of these antibodies observed between different animals. Chromatography of antisera on DEAE-Sephadex, followed by gel diffusion tests interpreted as described above, showed an increase in the ratio of virus particle-reactive to protein-reactive antibodies in fraction B and a decrease in fraction C. Van Regenmortel (1966) lists three possible explanations for the presence of two precipitin bands in immuno-diffusion tests involving virus antigens. The virus particle and the protein subunit may share some identical epitopes (antigenic sites on the surface of the antigens) and possess unrelated ones or they share related epitopes differing in their antibody combining capacity. The third alternative is that there is no antigenic relationship between the virus particle and the protein subunit. Regardless of the interpretation of the presence of two precipitin bands, our results demonstrated that the protein-reactive antibodies (detected in the precipitin band near the antiserum well) were predominantly of the fraction C type and the virus particle-reactive antibodies (detected in the precipitin band near the antigen well) were predominantly of the fraction B type.

Sela et al. (1963) showed that the distribution of antibodies between fractions B and C
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was a function of the net electrical charge on the inducing antigen. However, our system, involving two antigens in a state of equilibrium, is more complex. If the virus particle and its subunit protein share related epitopes it cannot be assumed that protein-reactive antibodies were completely protein-induced, or that virus particle-reactive antibodies were completely virus particle-induced. However, if there is no antigenic relationship between virus particle and subunit, the above assumption can be made. Furthermore in view of Sela’s evidence for the correlation between charge of the antigen and distribution of antibody between fraction B and C, it is unlikely that there would have been any separation of antibody specificities if there had not been two separate antigens.

The virus particles of both TCV and AMV had greater electrophoretic mobilities than their respective proteins, showing that the virus particles have a greater surface charge density (net charge per unit surface area), but not necessarily a greater charge per unit mass. For example, the negative mobility of polymerized A protein of TNV is three times that of depolymerized A protein (Ansevin et al. 1964); yet acid-base titration results (Scheele & Lauffer, 1967) demonstrated that depolymerized A protein has a higher negative charge per unit mass than polymerized A protein. Upon depolymerization the surface area is increased enormously and this causes the ratio of net charge to surface area to decrease substantially even though the ratio of charge to mass increases (Scheele & Lauffer, 1967). However, the surface charge density of the antigen is probably more important than the net charge per unit mass in determining the surface charge density of the induced antibodies.

Although the protein subunit was much less negatively charged than the virus particle, it did not carry a net positive charge at pH 7.0. Thus the increase in concentration and proportion of protein reactive antibodies in fraction C appears to contradict the conclusion arrived at by Sela and co-workers that fraction C type antibodies were induced by basic antigens. However, protein-reactive antibodies more electronegative than virus-reactive antibodies have been detected by immuno-electrophoresis of antisera against Prunus necrotic ringspot virus (Allen & Tremaine, 1965), foot-and-mouth disease virus (Brown, 1958) and alfalfa mosaic virus (Moed & Veldstra, 1968). It is possible that the preferential induction of fraction C-type antibodies is a general property of virus subunit proteins.

REFERENCES


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