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Purification and some Properties of Rabbit Anti-Ovalbumin

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Unlike previous reports that the ovalbumin–anti-ovalbumin complex did not dissociate completely in acid media, our results showed complete dissociation of the complex both in 1.2M-acetic acid, pH2.3, and in KCl–HCl, pH2.2, *I* 0.06. Thus Sephadex chromatography of the solution obtained by dissolving the antigen–antibody precipitate in these media repeatedly gave two peaks corresponding to anti-ovalbumin and ovalbumin. Further, gel-diffusion and immunoelectrophoresis experiments showed that the phosphate groups of ovalbumin are not involved in the antigenic sites. The antibody thus purified was more easily precipitated than previous preparations. The molecular weight and Stokes radius of the antibody were calculated from its gel-filtration behaviour and were found to be 148000 and 4.8nm respectively. The molecular weight determined by sodium dodecyl sulphate–polyacrylamide gel electrophoresis was essentially similar (about 0.7% lower).

The crucial step in the isolation and purification of antibody is the dissociation of specific antigen–antibody complex under conditions which give maximum recovery of the antibody without detectable loss of biological activity. Since the antigen–antibody complex is stabilized by non-covalent interactions, it could be dissociated at either acidic (Haurowitz *et al.*, 1948; Singer *et al.*, 1960; Givol *et al.*, 1962; Kavai *et al.*, 1966; Tarkhanova, 1966; Kierszenbaum & Dandliker, 1968) or alkaline (Liu & Wu, 1938) pH values. Previous studies (Kleinschmidt & Boyer, 1952; Singer *et al.*, 1955) demonstrated that the stability of the ovalbumin–anti-ovalbumin complex decreased on decreasing the pH. Several attempts were made to dissociate the ovalbumin–anti-ovalbumin complex in acid media over the pH range 1.8–2.4, but the dissociation was invariably incomplete (Givol *et al.*, 1962; Kavai *et al.*, 1966; Tarkhanova, 1966; Kierszenbaum & Dandliker, 1968). In the present paper we report complete dissociation of the ovalbumin–anti-ovalbumin complex both in KCl–HCl, pH2.2, *I* 0.06, and in 1.2M-acetic acid, pH2.3. The antibody thus obtained was characterized in terms of its size and hydrodynamic behaviour and was found to be biologically more active, as judged by ease of precipitation, than the preparations obtained by previous workers (Haurowitz *et al.*, 1948; Kavai *et al.*, 1966).

Experimental

Materials

Marker proteins listed in Table 1 were from Sigma Chemical Co. (St. Louis, Mo., U.S.A.) unless otherwise stated. The chemicals used in sodium dodecyl sulphate–polyacrylamide-gel electrophoresis were: acrylamide (E. Merck, Darmstadt, Germany); *NN'*-methylenebisacrylamide and *NNN'N'*-tetramethylenediamine (Fluka A.G., Chemische Fabrik, Bucks, Switzerland); ammonium persulphate and sodium azide (Riedel-De Haen A. G., Seelze-Hannover, Germany); sodium dodecyl sulphate and Bromophenol Blue (BDH, Poole, Dorset, U.K.); Coomassie Brilliant Blue (Sigma Chemical Co.); 2-mercaptoethanol (Calbiochem, Los Angeles, Calif., U.S.A.); iodoacetamide (Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K.); and urea, methanol, acetic acid, monobasic and dibasic sodium phosphate were all reagent grade. Sephadex and Blue Dextran were purchased from Pharmacia Fine Chemicals (Uppsala, Sweden).

Methods

Rabbit antiserum was raised against ovalbumin as described by Ansari & Salahuddin (1973). Quantitative precipitin titration, immunodiffusion and immunoelectrophoresis were performed by standard procedures (Kabat & Mayer, 1961).

Sodium dodecyl sulphate–polyacrylamide-gel electrophoresis. For the determination of molecular weight, sodium dodecyl sulphate–polyacrylamide-gel electrophoresis was carried out as described by Dunker & Rueckert (1969). All concentrations in this experiment as well as in others to be described below are given in weight/volume (w/v) unless otherwise stated. The proteins were dissolved to a concentration of about 2mg/ml in 0.01M-sodium phosphate buffer, pH7.5, containing 0.02% NaN_3, 6M-urea, 1%

sodium dodecyl sulphate, 0.0005% Bromophenol Blue and 1% (v/v) 2-mercaptoethanol. In two cases (bovine serum albumin and rabbit anti-ovalbumin), where cleavage and reshuffling of disulphide bonds were to be prevented, 2-mercaptoethanol was replaced by 0.002M-iodoacetamide. All protein solutions were incubated at room temperature for 24h before being electrophoresed. Protein solutions containing 25–50μg of protein were applied on top of the freshly prepared gels (approx. 6mm×95mm) containing 7.5% acrylamide, 0.203% methylenebisacrylamide, 0.1% sodium dodecyl sulphate, 0.015% ammonium persulphate, 0.08% (v/v) *NNN'N'*-tetramethylenediamine and 0.01M-sodium phosphate buffer, pH7.5. Electrophoresis was carried out at 8mA/gel in 0.01M-sodium phosphate buffer, pH7.5, containing 0.1% sodium dodecyl sulphate. After electrophoresis the gels were taken out and the position of the tracer dye was marked by inserting a piece of thin wire. Protein bands were stained with Coomassie Brilliant Blue (Weber & Osborn, 1969) and destained by diffusion.

Gel-filtration. For the determination of Stokes radius and molecular weight of anti-ovalbumin a column (3.1cm×54.5cm) of Sephadex G-200 was prepared in NaOH–succinic acid buffer, pH5.0, *I* 0.11, containing 0.02% NaN_3. The sample (2–20mg) was applied to the column and eluted with the equilibrating buffer at a flow rate of 1ml/h per cm^2 in 3ml fractions. Since the precision of the gel-filtration results depends partly on the uniformity of the fraction size, the latter was carefully maintained.

Purification of the antibody. The optimum amount of ovalbumin, as determined by quantitative precipitin titration, was incubated for 2h at 45°C (Ansari & Salahuddin, 1973) with the antiserum whose complements were previously inactivated by heating to 55°C for 30min. The suspension was centrifuged and the precipitate washed three to five times with cold 0.9% NaCl and finally dissolved in either 1.2M-acetic acid or KCl–HCl, pH2.2, *I* 0.06, by keeping the precipitate in the dissociating medium for about 14h at room temperature. Sephadex column chromatography of the solution gave two peaks; the fractions containing the antibody were pooled and concentrated.

Results

Sigma ovalbumin used for the immunization of rabbits gave two peaks on columns of both Sephadex G-200 and G-75. The elution patterns of the major and minor (7–9%) peaks were suggestive of the monomeric (mol.wt. 43000) and dimeric (mol.wt. 86000) forms of the protein respectively. Similar observations have been made by Holt & Creeth (1972) on another ovalbumin preparation. A single band was obtained on sodium dodecyl sulphate–polyacrylamide-gel electrophoresis of the protein antigen. These observations suggested size homogeneity of the protein preparation. Three types of ovalbumin molecules, which are identical in all respects except in their phosphate contents (two, one or no phosphate groups) have been recognized and designated as A_1, A_2 and A_3 respectively (Longsworth *et al.*, 1940; MacPherson *et al.*, 1949; Cann, 1949; Perlmann, 1952). Further, ovalbumin also exhibits heterogeneity owing to genetic variations causing single amino acid replacement, asparagine of class A ovalbumins being replaced by aspartic acid in class B ovalbumins (Wiseman *et al.*, 1972). Thus ion-exchange column chromatography of an ovalbumin preparation is expected to give four peaks. However, chromatography of Sigma ovalbumin on CM-cellulose column gave only three peaks, corresponding to the three fractions A_1, A_2 and A_3 as found by Rhodes *et al.* (1958). This precluded the possibility

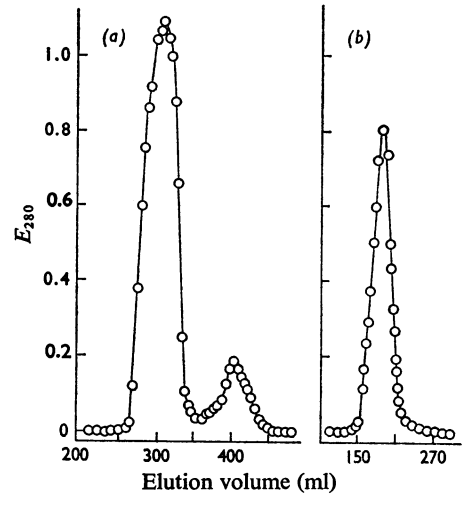


Fig. 1. *Typical chromatographic profiles of dissociated ovalbumin–anti-ovalbumin complex and of purified anti-ovalbumin*

(*a*) Chromatography on a column (3cm×120cm) of Sephadex G-75 of the ovalbumin–anti-ovalbumin complex dissociated in KCl–HCl, pH2.2, *I* 0.06; 36ml of the solution containing 1.2g of protein was applied and eluted with KCl–HCl at a flow rate of 30ml/h. (*b*) Chromatography of the purified anti-ovalbumin on a column (3.1cm×54.5cm) of Sephadex G-200 in NaOH–succinic acid buffer, pH5.0, *I* 0.11, containing 0.02% NaN_3; 10mg of the protein in 2.5ml of the buffer was applied to the column and run at a flow rate of 25ml/h. All protein concentrations were determined by measuring u.v. absorption at 280nm.

Table 1. *Proteins used in gel-filtration and gel-electrophoresis experiments*

V_e/V_0, ratio of elution volume to void volume; K_d, distribution coefficient; $K_{av.}$, available distribution coefficient; R_m, relative mobility with respect to the movement of the tracer dye; ‡, molecular weights used by Weber & Osborn (1969); §, calculated from the amino acid sequence, Brew *et al.*, (1967); ||, see William & Rajagopalan (1966); ¶, see Tanford (1968); **, calculated from the molecular weight of the monomer; ††, see Edelman & Gall (1969). Values for Stokes radius are from Andrews (1970).

Protein	10^{-3} × mol.wt.	Stokes radius (nm)	V_e/V_0	K_d	$K_{av.}$	R_m
(1) Cytochrome *c*	11.7‡	1.64	2.589 ± 0.001	0.8300 ± 0.0088	0.7897 ± 0.0076	0.903 ± 0.008
(2) Ribonuclease	13.7‡	1.92	2.585	0.8289	0.7870	—
(3) Lysozyme	14.4§	—	—	—	—	0.875 ± 0.007
(4) Haemoglobin*	15.5‡	—	—	—	—	0.850 ± 0.010
(5) Trypsin	23.3‡	—	—	—	—	0.760 ± 0.007
(6) Chymotrypsinogen†	25.7‡	2.24	2.319	0.6966	0.6619	0.750 ± 0.008
(7) Yeast alcohol dehydrogenase*	37.0‡	—	—	—	—	0.670 ± 0.006
(8) Pepsinogen†	40.0||	—	—	—	—	0.665 ± 0.005
(9) Ovalbumin	43.0‡	2.73	2.021	0.5393	0.5125	0.635 ± 0.010
(10) Catalase*	60.0‡	—	—	—	—	0.565 ± 0.004
(11) Bovine serum albumin	69.0¶	3.55	1.867 ± 0.006	0.4530 ± 0.0063	0.4304 ± 0.0061	0.540 ± 0.006
(12) Bovine serum albumin-dimer	138.0**	4.30	1.550 ± 0.007	0.2864 ± 0.0023	0.2721 ± 0.0040	0.400 ± 0.005
(13) Bovine IgG	150.0††	5.30	1.461 ± 0.004	0.2380 ± 0.0020	0.2262 ± 0.0019	—
(14) Bovine serum albumin-trimer	207.0**	—	—	—	—	0.320 ± 0.015
(15) Anti-ovalbumin	149.0	4.82	1.501 ± 0.008	0.2560 ± 0.0040	0.2478 ± 0.0034	0.385 ± 0.009

* Molecular weights of subunits have been given for these proteins.
† From Nutritional Biochemical Corporation (Cleveland, Ohio, U.S.A.)

of the existence of any genetic variants in the antigen preparation.

Plate 1 shows that ovalbumin as well as its individual fractions A_1, A_2 and A_3 gave single sharp precipitin bands in immunoelectrophoresis. Further, a single sharp precipitin line was obtained with ovalbumin in agar double diffusion, which fused completely with the lines given separately by fractions A_1, A_2 and A_3 showing no spurs or intersections. It therefore follows that the three fractions of ovalbumin are immunologically indistinguishable and that the protein antigen was immunologically homogeneous.

Purification

When the solution obtained by dissolving ovalbumin–anti-ovalbumin precipitate in KCl–HCl, pH2.2, was chromatographed on Sephadex G-75, G-100 or G-200 columns, only two peaks were obtained in each case (Fig. 1*a*), which by immunological and hydrodynamic criteria represented anti-ovalbumin and ovalbumin. Similar gel-filtration behaviour was observed when the complex was dissociated in 1.2M-acetic acid, pH2.3. Rechromatography of anti-ovalbumin on Sephadex G-75 in KCl–HCl or in 1.2M-acetic acid, as well as on Sephadex G-200 columns in succinate buffer, pH5.0, I 0.11, gave a single symmetrical peak. A typical chromatogram of the anti-ovalbumin is shown in Fig. 1(*b*). These observations demonstrated that the dissociation of the ovalbumin–anti-ovalbumin complex was complete both in KCl–HCl and in 1.2M-acetic acid. Further, it was noticed that such acid treatments did not inactivate the antibody, which was found to be 98% precipitable by the homologous antigen.

Sodium dodecyl sulphate–polyacrylamide-gel electrophoresis

Sodium dodecyl sulphate–polyacrylamide-gel electrophoresis of proteins was generally carried out in quadruplicate on 7.5% gels by using the same batch of solution of acrylamide and methylenebisacrylamide in the ratio 37:1 (w/w), and the results are shown on Plate 2. The relative mobility, R_m, of each protein could be measured with a precision of 2% (Table 1), which would introduce an error of about 4% in the estimation of the molecular weight. A least-squares analysis of the data fitted the equation:

$$\log M = 5.9807 - 2.1030\, R_m \qquad (1)$$

where M denotes the molecular weight of the protein polypeptide. As shown in Fig. 2 the plot of $\log M$ versus R_m is linear over the molecular-weight range 11 700–207000 and is devoid of any inflexion of the type observed by Dunker & Rueckert (1969) for 5% gels. The reduced anti-ovalbumin in sodium dodecyl sulphate–polyacrylamide gels moved as two bands, corresponding to H* and L chains of anti-ovalbumin; their molecular weights computed from their relative mobilities by eqn. (1) were 49800±1200 and 24700±1000 respectively. The maximum error involved in the measurement of molecular weight was about 5%, which is much less than that (about 10%) found by Björk & Tanford (1971*a*) for H chain by using sedimentation equilibrium. The unreduced anti-ovalbumin in sodium dodecyl sulphate–polyacrylamide gels moved as a single band corresponding to a molecular weight of 149000±7000. This value is identical with the molecular weight calculated from the individual molecular weights of H and L chains of anti-ovalbumin.

Gel chromatography

For the determination of molecular weight and Stokes radius of the antibody, gel-filtration data in Table 1 for marker proteins and anti-ovalbumin were obtained under identical conditions. Duplicate or triplicate measurements of V_e/V_o agreed within ±0.7%, which would introduce an error of about 2% in the evaluation of K_d and K_{av}. The latter uncertainty would cause insignificant error in the determination of Stokes radius (less than 0.8%) and molecular weight (2.5%) of the antibody. It should

* Abbreviations: IgG, immunoglobulin G; H and L chains, heavy and light chains of IgG.

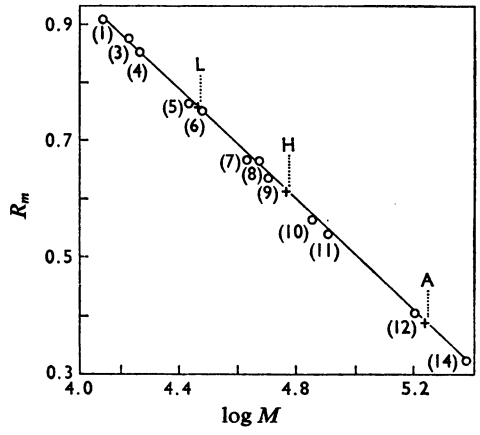
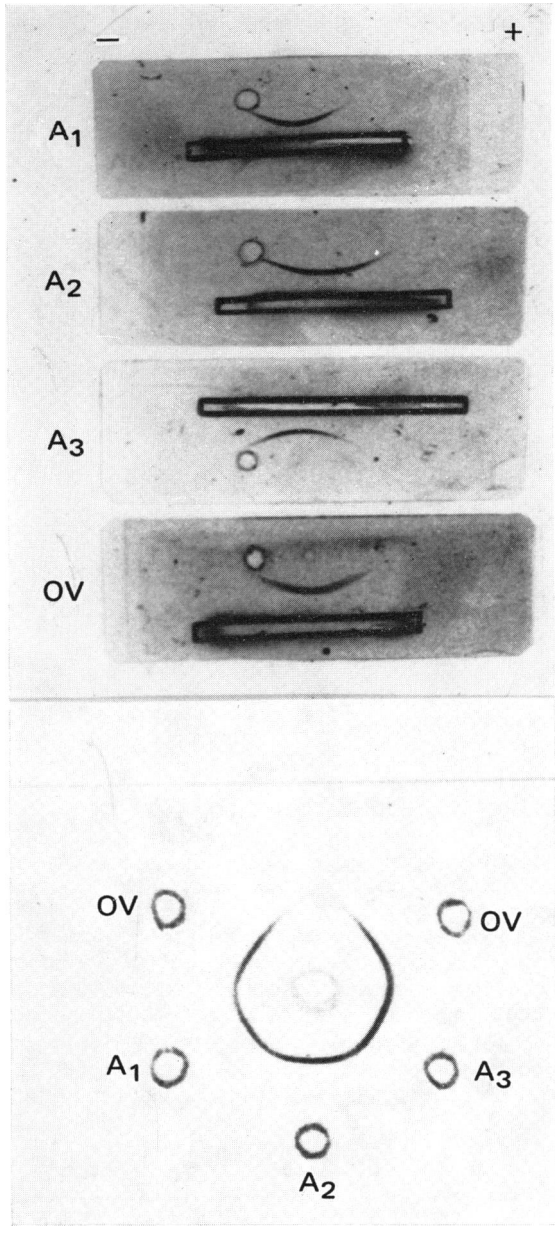


Fig. 2. *Sodium dodecyl sulphate–polyacrylamide-gel electrophoresis of proteins plotted as log M versus relative mobility*

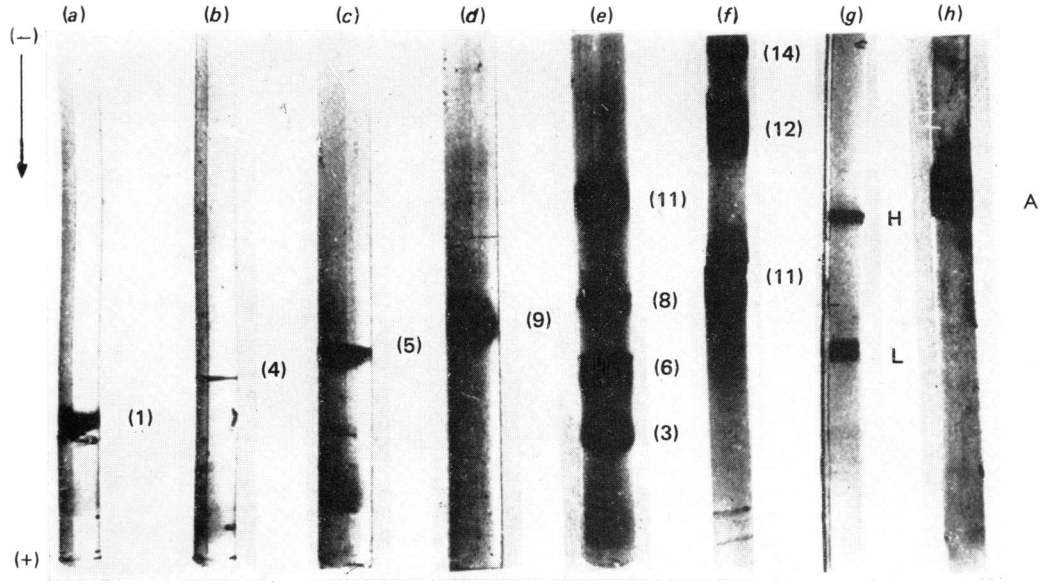
Experimental conditions were as described for Plate 2. Arabic numerals refer to the serial number of the proteins in Table 1. A, anti-ovalbumin; L and H, light and heavy chains of anti-ovalbumin.



EXPLANATION OF PLATE 1

Immunodiffusion and immunoelectrophoretic profiles of ovalbumin (OV) and its fractions A_1, A_2 and A_3 against the purified anti-ovalbumin

In immunoelectrophoresis (top four) the wells contained the antigen and the troughs contained the antibody. In the immunodiffusion plate (bottom) the peripheral wells contained ovalbumin and its three fractions and the central well contained the antibody.



EXPLANATION OF PLATE 2

Sodium dodecyl sulphate–polyacrylamide-gel electrophoretic patterns of proteins (25–50 μg) *on* 7.5% (*w*/*v*) *polyacrylamide gels* (*approx.* 6 *mm* × 95 *mm*) *containing* 0.1% *sodium dodecyl sulphate*

The proteins were dissolved at a concentration of about 2mg/ml in 0.01M-sodium phosphate buffer, pH7.5, containing 0.02% NaN_3, 6M-urea, 1% sodium dodecyl sulphate, 0.0005% Bromophenol Blue and 1% 2-mercaptoethanol (gels *a*, *b*, *c*, *d*, *e* and *g*) or 0.002M-iodoacetamide (gels *f* and *h*). All protein solutions were incubated at room temperature for 24h before being electrophoresed by using 0.01M-sodium phosphate buffer, pH7.5, at 8mA/gel. Gels (*c*), (*d*) and (*e*) were photographed after putting them in glass tubing; other gels were photographed naked. Position of the tracer dye in each case was different. Arabic numerals refer to the serial numbers of the proteins noted in Table 1. A, Anti-ovalbumin; L and H, light and heavy chains of anti-ovalbumin.

be mentioned that the gel-filtration behaviour of ovalbumin exhibits concentration-dependence, represented by the equation (Ackers, 1970):

$$K_d = K_d^0 (1 + Yc) \quad (2)$$

where K_d^0 is the limiting value of K_d at infinite dilution, c is the protein concentration in g/100ml, and Y is a constant having a value of 0.001–0.01 depending on the protein and the gel. However, the correction needed in the value of K_d for ovalbumin was computed to be too low (about 0.14%) to be of any significance at the protein concentration of 0.12% used in the present experiments.

The gel-filtration data for marker proteins were analysed by various procedures suggested by Porath (1963), Laurent & Killander (1964), and Ackers (1967), and were found to fit the following equations obtained by the method of least-squares:

$$\log M = 6.633 - 0.975\,(V_e/V_0) \quad (3)$$

Porath (1963):

$$M^{\frac{1}{3}} = 114.039 - 96.1919\,K_d^{\frac{1}{3}} \quad (4)$$

Laurent & Killander (1964):

$$(-\log K_{av.})^{\frac{1}{2}} = 0.0142\,r + 0.0985 \quad (5)$$

Ackers (1967):

$$erfc^{-1}\,K_d = 0.02\,r - 0.17 \quad (6)$$

where r is the Stokes radius and $erfc^{-1}\,K_d$ is the inverse error function complement of K_d. The results, shown graphically in Fig. 3, suggest that the gel-filtration behaviour of the proteins listed in Table 1 are as expected on the basis of the various semi-theoretical treatments of gel chromatography described above (eqns. 3–6). The Stokes radius and molecular weight of anti-ovalbumin were calculated by using the above equations and the data in Table 1. The value of molecular weight from eqn. (3) is

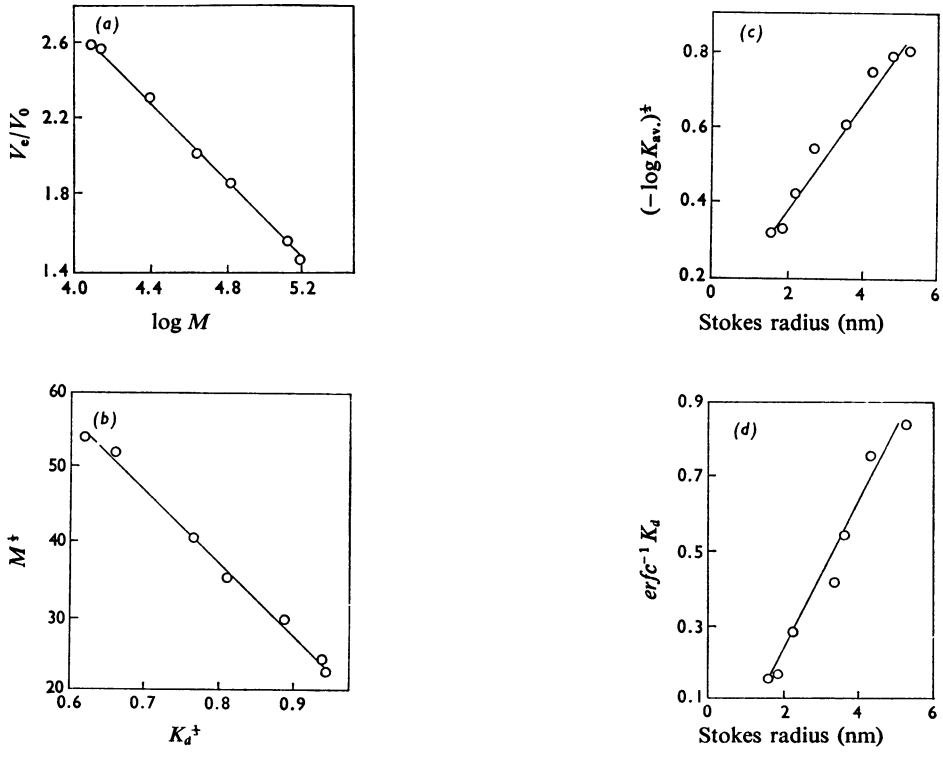


Fig. 3. *Plots of gel-filtration data of proteins according to different theories of gel-filtration*

The proteins (2–20mg) were run separately at a flow rate of 1ml/h per cm² on a column (3.1cm×54.5cm) of Sephadex G-200 equilibrated with NaOH–succinic acid buffer, pH5.0, I 0.11, containing 0.02% NaN_3. (*a*) Plot of V_e/V_0 versus log$M$; (*b*) plot by the method of Porath (1963); (*c*) plot by the method of Laurent & Killander (1964); (*d*) plot by the method of Ackers (1967).

148000±2500, which is identical, within experimental error, with 148700±2400 calculated from eqn. (4). Similarly, the values of Stokes radius of the antibody computed from eqns. (5) and (6) were respectively 4.79±0.02nm and 4.84±0.01nm, which are experimentally indistinguishable. Thus the molecular weight of the antibody from its gel-filtration behaviour is essentially identical (about 0.7% lower) with that measured by sodium dodecyl sulphate–polyacrylamide-gel electrophoresis.

Discussion

Published results (cited in Boyd, 1956) on the antigenicity of ovalbumin suggest at least five antigenic determinants on the ovalbumin molecule. Our results have shown that the three ovalbumin fractions, namely A_1, A_2 and A_3, are immunologically identical. This would mean that none of the antigenic determinants contains a phosphate group.

The ovalbumin–anti-ovalbumin complex, even in the soluble state, dissociated in acid at pH2.4 (Singer *et al.*, 1955). However, complete dissociation of the complex in 1M-acetic acid, pH2.4 (Kavai *et al.*, 1966), glycine–H_2SO_4 buffer, pH2.4 (Tarkhanova, 1966), and in glycine–HCl buffer, pH2.25 (Kierszenbaum & Dandliker, 1968) could not be achieved. Consequently, when the solutions obtained by dissociating the ovalbumin–anti-ovalbumin precipitate in the three dissociating media were chromatographed on Sephadex G-200 columns, three peaks representing the soluble complex, anti-ovalbumin and ovalbumin respectively were observed (Kavai *et al.*, 1966; Tarkhanova, 1966; Kierszenbaum & Dandliker, 1968). Our data, however, clearly demonstrate complete dissociation of the ovalbumin–anti-ovalbumin complex both in 1.2M-acetic acid, pH2.3, and in KCl–HCl, pH2.2, I 0.06. The ovalbumin–anti-ovalbumin precipitate dissolved in these solutions gave only two peaks corresponding to anti-ovalbumin and ovalbumin on Sephadex G-200, G-100 or G-75 columns. The inability of glycine–HCl buffer of the same pH (2.25) to dissociate the antigen–antibody complex completely (Kierszenbaum & Dandliker, 1968) suggests that glycine is not as effective a dissociating medium as either acetic acid or KCl. It may be mentioned that Givol *et al.* (1962) could not show dissociation of the ovalbumin–anti-ovalbumin complex on a Sephadex G-75 column in 0.02M-HCl–0.15M-NaCl, pH1.8, which is even lower than the pH used in the present studies. The discrepancy may be ascribed either to lesser effectivity of NaCl in comparison with KCl or to the relatively higher ionic strength (about 0.15) used by Givol *et al.* (1962). Here it is instructive to recall our previous findings (Ansari & Salahuddin, 1973) showing maximum precipitation of ovalbumin–anti-ovalbumin complex at an ionic strength of 0.15.

The antibody purified by the procedure described in the present paper was biologically more active than other preparations (Haurowitz *et al.*, 1948; Kavai *et al.*, 1966) and was 98% precipitable by the antigen.

Since H chain of anti-ovalbumin has a marked tendency to undergo aggregation in aqueous solution, a quantitative estimate of its molecular weight in the 'native' state is difficult. The molecular weights of H and L chains of the anti-ovalbumin measured by sodium dodecyl sulphate–polyacrylamide-gel electrophoresis were 49800 and 24700 respectively, which are in good agreement with the molecular weights of the corresponding chains in normal rabbit IgG (Björk & Tanford, 1971*a*, *b*; Marler *et al.*, 1964; Small & Lamm, 1966). By summation, the molecular weight of the antibody is 149000. An essentially identical value (148000) of the molecular weight of the antibody was determined by Sephadex gel chromatography. The Stokes radius of the antibody calculated from its gel-filtration behaviour was 4.8nm, which is about 9% lower than that measured for normal bovine IgG.

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