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Identification of the immunoglobulin class active in the Rose Bengal plate test for bovine brucellosis

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SUMMARY

The antibodies active in the Rose Bengal plate test (RBPT) for bovine brucellosis have been studied. The results of fractionation experiments showed that RBPT activity was associated with fractions containing immunoglobulin of the IgG_1 class; other immunoglobulin classes were inactive in this respect although active in other tests. These results were confirmed by inhibition tests with specific antisera and by elution of the antibody from agglutinated RBPT antigen.

The major proportion of the serum complement-fixing activity was also present in the IgG_1 fraction and it is suggested that the RBPT and CF reactions are probably mediated by the same antibodies.

INTRODUCTION

The serum agglutination (SA) test using standardized antigen has been used extensively for the diagnosis of brucellosis in man and domestic animals. However, it suffers from the disadvantage that false positive reactions sometimes occur. These may be the result of cross-reactions between antigens of *Brucella* spp and unrelated organisms, for example *Yersinia enterocolitica* (Ahvonen, Jansson & Aho, 1969; Corbel & Cullen, 1970) or they may result from the presence of non-specific agglutinins distinct from antibodies, which are present in certain bovine sera (Hess, 1953*a*, *b*). In an attempt to differentiate specific brucella agglutinins from these non-specific factors, Rose & Roepke (1957) introduced a modification of the plate-agglutination test in which the antigen was buffered at pH 4·0 immediately before use. They observed that at this pH, agglutination of *Br. abortus* cells by the non-specific agglutinins of bovine serum was inhibited, whereas the activity of specific brucella antibodies was largely unaffected. Subsequent experience showed that the acid plate-agglutination test was of value as a supplemental test in screening field samples of serum for brucella-specific agglutinins (Lambert & Amerault, 1962*a*, *b*).

More recently, a modification of the acid plate-agglutination test, employing a suspension of *Br. abortus* cells stained with Rose Bengal dye and buffered at pH 3·65 was introduced by the United States Department of Agriculture, National Animal Diseases Laboratory, as the basis of a card test for bovine brucellosis. In field trials this test was found to be a more accurate indicator of infection than the SAT (Nicoletti, 1967).

Using the Rose Bengal antigen prepared according to USDA procedures as the basis of a plate test for examining serum samples, Morgan (1969) and Morgan, MacKinnon, Lawson & Cullen (1969) obtained results which correlated well with those of the complement-fixation (CF) test. Comparison of the results of the combined CF and SA tests, interpreted as laid down for the Brucellosis (Accredited Herds) Scheme, with those of the Rose Bengal plate test (RBPT) showed agreement for 90·8 % of sera. Later results (Davies, 1971) showed agreement for 97 % of sera. At present the RBPT is in routine use for screening field samples of cattle sera in connexion with the Brucellosis Incentives Scheme.

However, although the relationship between the results of the CF test and the RBPT suggested that similar antibodies may be involved in both tests, little specific information was available on this point. This study was undertaken, therefore, with the object of characterizing the antibodies involved in the RBPT and determining their relation to the results of other diagnostic tests for brucellosis.

MATERIALS AND METHODS

Sera used

Bovine sera. High titre serum was collected from cattle infected with virulent field strains of *Br. abortus*. For fractionation purposes batches of serum from three different animals were pooled. Serum was also obtained from cattle vaccinated with *Br. abortus* Strain 19 vaccine. Samples were collected at various intervals after vaccination.

Brucella-negative serum was obtained from bullocks kept under Brucella-free conditions.

All serum samples were membrane filtered and stored at $-20°$ C. until required.

Rabbit antisera. Rabbit antiserum to bovine serum proteins was produced by repeated subcutaneous and intramuscular injection of New Zealand white rabbits with bovine serum emulsified in Freund's complete adjuvant. The animals were exsanguinated after a course of injections extending over 4 months. Rabbit antiserum to bovine γ-globulin was produced by a similar process except that γ-globulin fractions prepared according to Keckwick (1940) were used as antigen.

Class-specific antisera to IgG_1, IgG_2 and IgM were prepared essentially according to Porter & Noakes (1970). They were rendered specific by absorption with foetal calf serum and heterologous immunoglobulin.

Serological tests

The procedures used for the SAT, Coombs antiglobulin, RBPT, quantitative RBPT (QRBPT) and immunodiffusion tests have been described or referred to previously (Corbel & Cullen, 1970). CF tests were done according to MacKinnon (1963). Rivanol and heat inactivation tests were performed as described by Morgan (1967).

Disulphide bond reduction tests

For the test, 0·2 ml. of serum was incubated with an equal volume of 0·02 M dithiothreitol (DTT; British Drug Houses, Poole) in phosphate buffered saline (PBS; 0·15 M-NaCl in 0·01 M phosphate pH 7·5) at 37° C. for 30 min. A volume of

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0·6 ml. of 0·15 M-NaCl was then added to the mixture and serial doubling dilutions made in the same diluent using a unit volume of 0·5 ml. After addition of an equal volume of standard *Br. abortus* agglutination suspension to each tube, the tests were incubated and read as for the SA test (WHO Monograph 19, 1953).

Immunoglobulin inhibiton tests

Specific immunoglobulins were removed from bovine serum or serum fractions by addition of 2 vol. of class-specific antiglobulin serum followed by incubation for 16 hr. at 4° C. Precipitates were removed by centrifugation at 20,000*g* for 30 min. and the samples examined by immunoelectrophoresis. If necessary the process was repeated until the specific precipitin arc could no longer be detected.

Serum fractionation procedures

Density gradient ultracentrifugation. A modification of the method of Cowan & Trautman (1965) was used. Gradients were formed in 15 ml. polycarbonate centrifuge tubes (MSE, London) by layering 3 ml. volumes of 65% saturated $NaNO_3$ over 5 ml. volumes of 75% saturated KBr. Samples of 2 ml. of serum were layered above the gradient and residual space was filled with liquid paraffin. Centrifugation was performed at an average relative centrifugal force of 90,000*g* for 24 hr. at 12° C. Fractions of 0·5 ml. volume were collected by aspiration. These were freed of gradient salt by dialysis against PBS and concentrated by ultra-filtration (Selecta Ultra-thimbles; Schleicher u. Schull, Germany).

Gel filtration. Essentially the method of Flodin & Killander (1962) was used. A column of Sephadex G200 (Pharmacia, Uppsala) 850 mm. × 25 mm. diameter, equilibrated with Tris (hydroxymethyl amino methane)–HCl buffer (Tris buffer; 1 M-NaCl; 0·1 M Tris-HCl, pH 8·0; NaN_3 0·01 M) was used. Samples of up to 5 ml. volume of serum were applied and eluted by upward development with the equilibrating buffer at a flow rate of 25 ml. per hour. Effluent was monitored for absorption of ultraviolet light at 280 nm. wavelength by a Uvicord II detector (LKB Produkter, Bromma) and fractions of 5 ml. volume collected automatically by an Ultro-Rac fraction collector (LKB Produkter, Bromma). Before testing, fractions were dialysed and concentrated as described for density gradient centrifugation.

Ion-exchange chromatography

This was performed on 300 mm. × 10 mm. columns of QAE-Sephadex-A50 (Pharmacia, Uppsala) equilibrated with 0·1 M phosphate buffer, pH 7·0. Volumes of 2·0 ml. of serum dialysed to equilibrium with the starting buffer were applied to the column and fractions eluted sequentially with 0·1 M, 0·2 M, 0·3 M, 0·4 M and 1·0 M-NaCl buffered at pH 7·0 with 0·01 M phosphate. Fractions were treated as described for gel filtration.

Preparative zone electrophoresis

This was done using 200 mm. × 50 mm. × 5 mm. blocks of cellulose acetate gel (Cellogel; Chemetron, Milan) equilibrated with barbital buffer, 0·05 *I*, pH 8·6. The procedure recommended by the manufacturers was followed throughout.

Volumes of 0·2 ml. of serum were applied to each block. After electrophoresis, blocks were sectioned tranversely into 10 mm. segments and fractions expressed from the gel and concentrated by ultra-filtration.

Immuno-adsorption and elution of antibody

Rose Bengal plate test antigen (prepared according to USDA, National Animal Diseases Laboratory, Diagnostic Reagents Manual 65c) was washed by two cycles of centrifugation in lactate buffer, pH 3·65, and one cycle of centrifugation in 0·1 M formate-HCl buffer, pH 1·0, and resuspended to its original volume in lactate buffer, pH 3·65.

Equal volumes of this suspension and pooled high titre bovine anti-*Br. abortus* serum were mixed and incubated at 4° C. for 16 hr. The agglutinated cells were sedimented by centrifugation at 2000*g* for 20 min. and the supernatant discarded. The sediment was resuspended in lactate buffer at pH 3·65 to the original volume and centrifuged again at 5000*g* for 20 min. The supernatant was discarded and the deposit resuspended in PBS and washed twice by centrifugation in this medium. Antibodies were eluted by resuspension of washed agglutinated cells in 0·15 M-NaCl buffered at pH 1·0 with 0·1 M formate-HCl buffer. After standing for 4 hr. at 4° C. the cells were removed by centrifugation at 15,000*g* for 30 min. The supernatant was neutralized by addition of NaOH and concentrated by ultra-filtration.

To check for possible non-specific adsorption of serum proteins an identical experiment was performed in parallel but using negatively reacting bovine serum.

Analytical methods

Immunoglobulin concentrations were measured by radial immunodiffusion (Mancini, Carbonara & Heremans, 1965) using antisera specific for bovine IgG_1, IgG_2 and IgM globulins.

Immunoelectrophoresis was performed essentially according to Scheidegger (1955). To identify unknown components, the interrupted trough method (Wieme, 1965) and a combination of immunoelectrophoresis and immunodiffusion were used. In the latter case immunoelectrophoresis of defined antigens was performed in the normal manner except that before addition of antiserum to the peripheral troughs an additional antigen well was cut at a distance of 10 mm. from the centre of the estimated position of the precipitin arc corresponding to the known component. This well was then filled with a sample of the unknown component, antiserum was added to the peripheral troughs and diffusion allowed to proceed in the usual manner. In some tests the positions of the defined and undefined samples were reversed.

Disk electrophoresis was performed according to Davis (1964). Thin-layer gel filtration on Sephadex G200 Superfine was done according to Morris (1964). Results were recorded by making imprints of the wet gel on cellulose acetate membrane and staining with 0·002% nigrosin in 2% acetic acid.

Estimations of total protein concentration were made spectrophotometrically according to Cullen & Corbel (1970) using pure bovine IgG_2 globulin as standard.

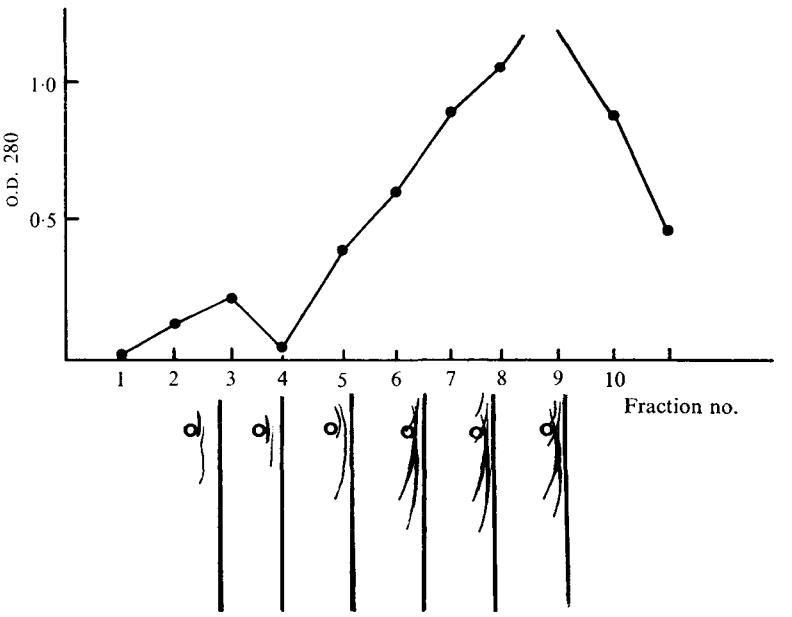

Fig. 1 Density gradient ultracentrifugation of bovine serum.

RESULTS

Fractionation of sera

Density gradient ultracentrifugation

Fractionation of high titre serum from infected cows produced the results shown in Fig. 1. The serological activities of the fractions are summarized in Table 1. Antibodies active in the SAT and antiglobulin tests were present in fast sedimenting and slowly sedimenting fractions and their distribution was related to that of the 19s and 7s immunoglobulins. The major part of the Coombs antibody was present in the 7s fractions. These also contained all detectable CF, RBPT and DTT-stable antibodies.

Fractionation of serum from cattle vaccinated with *Br. abortus* S19 vaccine produced results which varied with the interval after vaccination.

In the case of serum collected at 7 days after vaccination agglutinins were largely confined to the fast sedimenting fractions. Some Coombs antibody was however detected in both fast and slow fractions. No significant CF, RBPT or DTT-stable activity was detected in any fraction although the unfractionated serum had low titres of CF and RBPT antibodies (Table 1). When serum collected 22 days post-vaccination was fractionated by the same procedure the results obtained were very similar to those given by serum from infected animals. Again, agglutinins active in the SAT were present in both fast and slowly sedimenting fractions but the RBPT, CF and DTT-stable activities were confined to the slow fractions (Table 1). When serum collected from cattle vaccinated 3 years previously and having a low residual SAT and positive RBPT was fractionated in the same way, agglutinins were detected in fast sedimenting fractions only.

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Table 1. *Serological activity of fractions separated from bovine antisera to* Br. abortus *by density gradient centrifugation*

Sample	Reciprocal titres				
	SAT	Coombs test	DTT test	QRBPT	CFT
Pooled high titre serum	2560	> 5120	1280	1024	4000
Fraction no.					
1	< 10	10	< 10	< 1	4
2	< 10	20	< 10	< 1	4
3	10	40	< 10	4	4
4	40	160	20	8	20
5	160	640	80	160	80
6	320	2560	320	160	400
7	640	2560	320	320	1000
8	640	2560	640	320	1000
9	320	2560	160	160	200
10	80	640	40	32	40
Serum 7 days after S19 vaccine	160	320	20	4	40
Fraction no.					
1	< 10	< 10	< 10	< 1	< 2
2	10	10	< 10	< 1	< 2
3	40	40	< 10	< 1	< 2
4	80	80	< 10	< 1	4
5	10	20	< 10	1	10
6	10	20	10	2	4
7	10	20	10	2	2
8	10	20	< 10	1	2
9	< 10	10	< 10	< 1	< 2
10	< 10	< 10	< 10	< 1	< 2
Serum 22 days after S19 vaccine	640	2560	320	64	200
Fraction no.					
1	< 10	< 10	< 10	< 1	< 2
2	< 10	10	< 10	< 1	< 2
3	< 10	20	< 10	< 1	< 2
4	40	80	10	1	10
5	40	160	20	4	20
6	40	160	20	3	20
7	80	320	40	8	40
8	40	160	20	4	10
9	10	40	< 10	1	2
10	< 10	10	< 10	< 1	< 2
Serum ca. 3 years after S19 vaccine	40	80	< 10	1	< 2
Fraction no.					
1	< 10	< 10	< 10	< 1	< 2
2	< 10	10	< 10	< 1	< 2
3	40	80	< 10	< 1	< 2
4	10	10	< 10	< 1	< 2
5	10	10	< 10	< 1	< 2
6	< 10	< 10	< 10	< 1	< 2
7	< 10	< 10	< 10	< 1	< 2
8	< 10	< 10	< 10	< 1	< 2
9	< 10	< 10	< 10	< 1	< 2
10	< 10	< 10	< 10	< 1	< 2

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Table 2. *Fractionation of bovine sera by gel filtration on Sephadex G200*

Fraction no....	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
						(*a*) Serum from naturally infected cattle										
Absorption peak	0*	1	1	1	1	1	2	2	2	2	2	2	3	3	3	3
SAT	2560	< 10	< 10	10	10	< 10	20	40	160	160	40	20	< 10	< 10	< 10	< 10
Coombs	> 5120	< 10	< 10	10	10	40	40	160	640	1280	640	160	10	< 10	< 10	< 10
DTT	1280	< 10	< 10	< 10	< 10	< 10	20	160	640	640	320	160	< 10	< 10	< 10	< 10
QRBPT	1024	< 1	< 1	< 1	< 1	< 1	4	16	32	32	8	8	2	< 1	< 1	< 1
CFT	4000	< 2	< 2	< 2	2	10	20	80	200	200	100	40	2	< 2	< 2	< 2
						(*b*) Serum: 7 days after S19 vaccination										
Absorption peak	0*	1	1	1	1	1	2	2	2	2	2	2	3	3	3	3
SAT	160	< 10	20	40	20	10	10	< 10	< 10	10	< 10	< 10	< 10	< 10	< 10	< 10
Coombs	320	< 10	20	40	20	40	40	10	20	20	< 10	< 10	< 10	< 10	< 10	< 10
DTT	20	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10
QRBPT	4	< 1	< 1	< 1	< 1	< 1	< 1	< 1	< 1	< 1	< 1	< 1	< 1	< 1	< 1	< 1
CFT	20	< 2	< 2	< 2	< 2	< 2	< 2	< 2	< 2	< 2	< 2	< 2	< 2	< 2	< 2	< 2
						(*c*) Serum: 22 days after S19 vaccination										
Absorption peak	0*	1	1	1	1	1	2	2	2	2	2	2	3	3	3	3
SAT	640	< 10	< 10	< 10	< 10	10	20	40	80	80	80	80	< 10	< 10	< 10	< 10
Coombs	2560	< 10	10	20	40	40	80	320	640	640	320	320	< 10	< 10	< 10	< 10
DTT	320	< 10	< 10	< 10	< 10	< 10	10	20	40	40	40	40	< 10	< 10	< 10	< 10
QRBPT	64	< 1	< 1	< 1	1	1	2	8	16	16	16	4	< 1	< 1	< 1	< 1
CFT	200	< 2	< 2	< 2	4	4	10	20	20	40	40	40	< 2	< 2	< 2	< 2
						(*d*) Serum: 3+ years after S19 vaccination										
Absorption peak	0*	1	1	1	1	1	2	2	2	2	2	2	3	3	3	3
SAT	40	< 10	10	20	10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10
Coombs	80	< 10	10	20	10	10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10
DTT	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10
QRBPT	1	< 1	< 1	< 1	< 1	< 1	< 1	< 1	< 1	< 1	< 1	< 1	< 1	< 1	< 1	< 1
CFT	< 2	< 2	< 2	< 2	< 2	< 2	< 2	< 2	< 2	< 2	< 2	< 2	< 2	< 2	< 2	< 2

* Unfractionated serum.

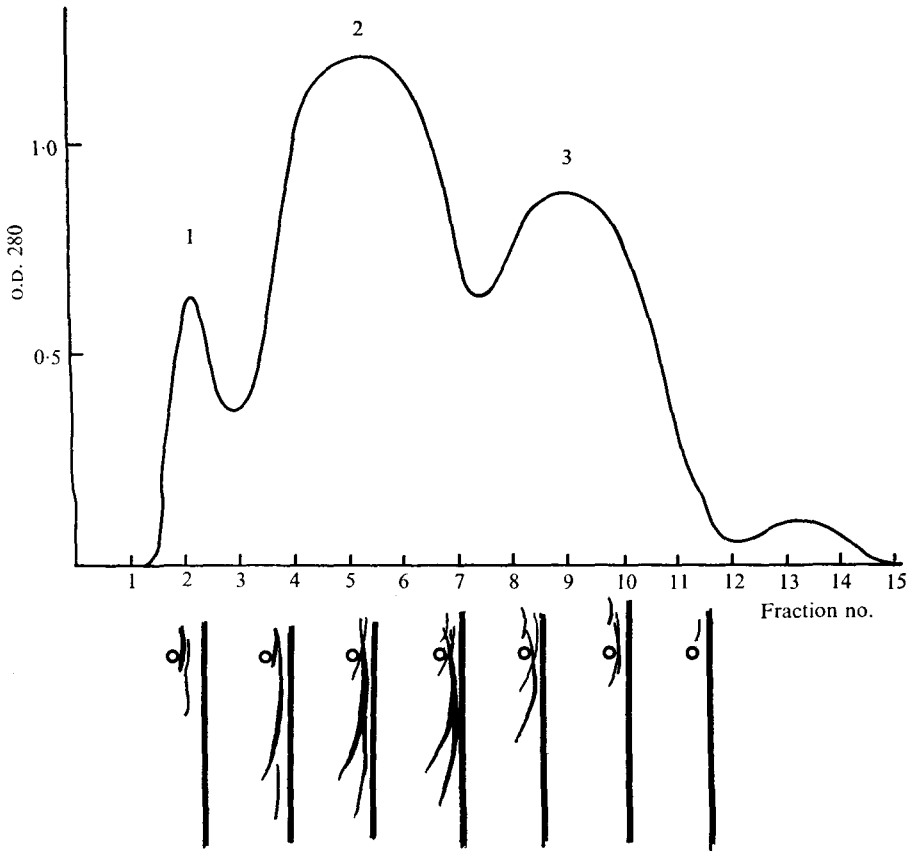

Fig. 2. Gel filtration of bovine serum on Sephadex G200.

No CF and RBPT activities were detected and the agglutinating activity was DTT-labile.

Gel filtration

Fractionation of high titre anti-*Br. abortus* serum from infected cattle produced an elution pattern as shown in Fig. 2. Antibody activity towards *Br. abortus* was present in fractions corresponding to absorption peaks 1 and 2. No antibody activity was detected in fractions corresponding to peak 3. Agglutinins active in the SAT were recovered from peak 1 and 2 fractions. Antibodies active in the DTT, Coombs and CF tests were restricted almost entirely to fractions corresponding to peak 2. Similarly, agglutinins active in the RBPT were only recovered from peak 2 fractions (Table 2).

Fractionation of serum samples from cattle vaccinated with *Br. abortus* S19 vaccine produced results varying with the time of collection of serum samples. In each case the elution profiles were similar to those shown in Fig. 2 but the antibody activities of the fractions varied. Serum collected 7 days after vaccination contained agglutinins restricted largely to peak 1 with only low titres of Coombs antibodies and no DTT-stable, CF or RBPT activity being detected in any fraction, although these activities were present to low titre in the unfrac-

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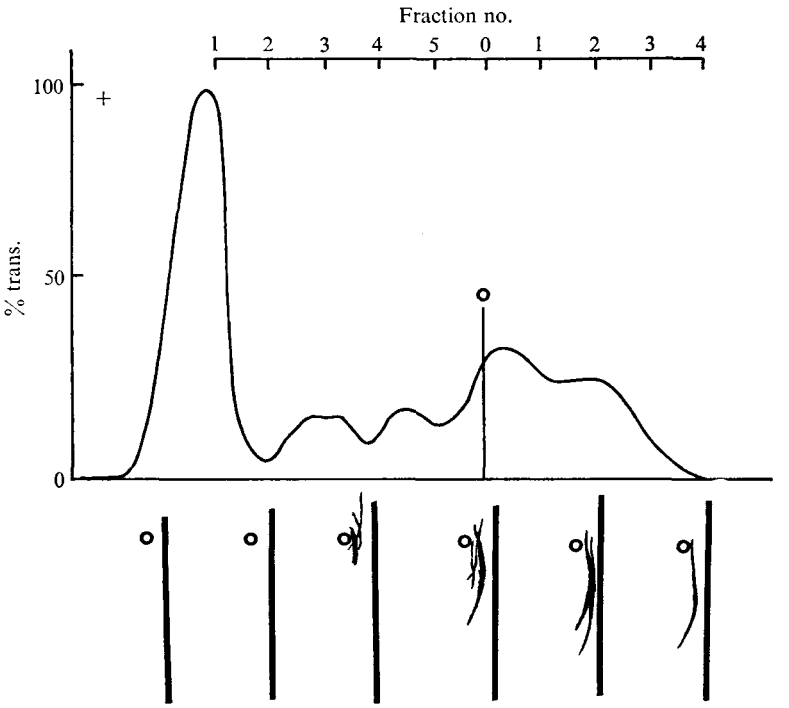

Fig. 3. Cellogel block electrophoresis of bovine serum.

tionated serum. Serum collected at 22 days produced a pattern similar to that of high titre serum from naturally infected animals. Agglutinins were present in fractions corresponding to peaks 1 and 2 but DTT stable agglutinins, CF and RBPT activity were restricted to peak 2 fractions. Some Coombs antibody was present in peak 1 fractions but the major part was in the peak 2 fractions (Table 2).

Serum with a low residual agglutinin titre pooled from animals vaccinated 3 years previously gave results similar to those of the 7 days serum. Agglutinins were restricted to peak 1 and the only other activity detected was Coombs antibody in low titre which was present in peak 1 and 2 fractions (Table 2). The RBPT was positive on undiluted serum but no activity was recovered in fractionated material.

A sample of negative serum fractionated as a control gave fractions inactive in all tests although the ultraviolet absorption profile was virtually identical with that of positive serum.

Preparative electrophoresis

Electrophoresis of high titre anti *Br. abortus* serum in cellulose acetate gel blocks produced the results shown in Fig. 3. Serological activity was present in fractions from the origin and cathodal to the origin. Agglutinating activity was present in γ_1 and γ_2 fractions. CF activity was restricted to the γ_1 fractions. DTT-stable antibodies and Coombs antibodies were present in γ_1 and γ_2 fractions but RBPT activity was restricted to the γ_1 region.

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Table 3. *Serological activity of fractions separated from bovine antisera to* Br. abortus *by zone electrophoresis*

Sample	Distance from origin (mm)	SAT	Coombs	DTT	QRBPT	CFT
Pooled high titre bovine serum	Unfractionated	2560	> 5120	1280	1024	4000
Fraction no.						
1	+50	< 10	< 10	< 10	< 1	< 2
2	+40	< 10	< 10	< 10	< 1	< 2
3	+30	< 10	< 10	< 10	< 1	< 2
4	+20	10	20	< 10	1	< 2
5	+10	80	640	40	8	80
6	0	160	1280	80	32	200
7	−10	160	1280	160	32	200
8	−20	80	640	40	8	40
9	−30	10	640	< 10	< 1	< 2
10	−40	< 10	10	< 10	< 1	< 2
Serum 22 days after S19 vaccine	Unfractionated	640	2560	320	64	200
Fraction no.						
1	+50	< 10	< 10	< 10	< 1	< 2
2	+40	< 10	< 10	< 10	< 1	< 2
3	+30	< 10	< 10	< 10	< 1	< 2
4	+20	< 10	10	< 10	< 1	< 2
5	+10	40	160	20	4	20
6	0	160	1280	80	16	40
7	−10	80	640	80	2	4
8	−20	10	640	< 10	< 1	2
9	−30	< 10	320	< 10	< 1	< 2
10	−40	< 10	10	< 10	< 1	< 2

No activity was recovered on fractionation of low titre sera from animals vaccinated with *Br. abortus* strain 19. Fractionation of serum collected 22 days after vaccination produced results qualitatively similar to those obtained with serum from naturally infected animals (Table 3).

Ion-exchange chromatography

Fractionation of high titre serum samples produced the results illustrated in Fig. 4. Agglutinating antibodies with Coombs activity and stable to DTT, but with no CF or RBPT activity, were eluted in fractions corresponding to peak 1. Immunoelectrophoresis showed that these fractions contained almost entirely IgG_2 immunoglobulin (Fig. 4). Antibodies with RBPT, Coombs, CF and agglutinating activity stable to DTT were recovered in fractions corresponding to peak 2. Peaks 3–5 contained agglutinins with Coombs activity. Those from peak 5 were partially susceptible to degradation with DTT. With the exception of peak 3 no CF or RBPT activity was present in these fractions (Table 4). Immunoelectrophoresis showed that peak 2 contained, *inter alia*, IgG_1 immunoglobulin. The other fractions were more heterogeneous and IgM and possibly IgA were present (Figure 4).

Fractionation of low titre sera from vaccinated animals did not produce useful

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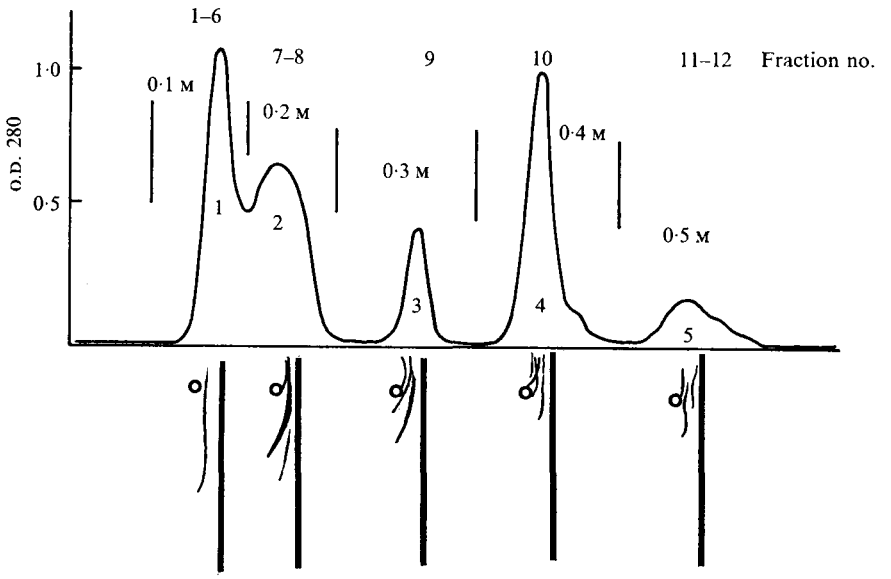

Fig. 4. Ion-exchange chromatography of bovine serum on QAE Sephadex

results. Fractionation of serum collected 22 days after vaccination produced results which were qualitatively identical with those obtained with high titre serum from infected animals (Table 4).

Examination of antibodies recovered by immuno-adsorption and elution

The eluates recovered from RBPT antigen by acid elution were examined for antibody activity in serological tests. The results summarized in Table 5 showed that eluates contained agglutinating, CF, RBPT, Coombs and DTT-stable antibodies. The RBPT and CF titres in relation to the SAT titres were higher than in the original serum. On diffusion against *Br. abortus* extracts (Corbel & Cullen, 1970) the eluates formed precipitin lines against the lipopolysaccharide component but showed only slight activity towards the intracellular antigens.

The eluates were characterized by disk electrophoresis, thin-layer gel filtration, immunodiffusion and immunoelectrophoresis. Disk electrophoresis followed by staining for protein, revealed a single major band in the γ-globulin region (Pl. 1; Fig. 1). Several faintly staining minor components of fast mobility were also detected but these were probably derived from the RBPT antigen. Thin-layer gel filtration demonstrated a single protein component with exclusion properties identical with those of bovine 7s globulin (Pl. 1; Fig. 2).

Immunoelectrophoresis of eluates showed a single precipitating component of γ_1 mobility. Precipitation occurred with antisera to bovine serum, bovine γ-globulin and specific antiserum to bovine IgG_1 globulin. No precipitation was produced by specific antisera to IgM or IgG_2 immunoglobulins nor by antiserum to bovine colostrum absorbed with bovine IgG_1 protein. Use of the interrupted trough technique showed fusion of the eluate precipitin arc with the IgG_1 arc of a bovine γ-globulin fraction containing IgG_1 and IgG_2 immunoglobulins (Pl. 1; Fig. 3).

Table 4. *Serological activity of fractions separated from bovine antisera to* Br. abortus *by ion-exchange chromatography on QAE-Sephadex*

Sample	Peak number	SAT	Coombs	DTT	QRBPT	CFT
Pooled high titre serum	Unfractionated	2560	5120	2560	1024	4000
Fraction no.						
1	1	20	1280	10	< 1	< 2
2	1	20	1280	10	< 1	< 2
3	1	20	1280	10	< 1	< 2
4	1	20	640	10	< 1	< 2
5		10	40	10	< 1	2
6		10	40	< 10	< 1	2
7	2	320	2560	160	32	200
8	2	640	5120	640	128	800
9	3	20	640	10	2	10
10	4	20	40	< 10	< 1	< 2
11	5	10	10	< 10	< 1	2
12	5	< 10	< 10	< 10	< 1	< 2
Serum 22 days after S19 vaccine	Unfractionated	640	2560	320	64	200
Fraction no.						
1	1	< 10	160	< 10	< 1	< 2
2	1	10	320	< 10	< 1	< 2
3	1	20	640	10	< 1	< 2
4	1	10	320	< 10	< 1	< 2
5		< 10	20	< 10	< 1	2
6		10	40	10	< 1	2
7	2	160	1280	80	16	200
8	2	160	1280	80	32	200
9	3	40	160	20	8	10
10	4	10	20	< 10	1	2
11	5	10	10	< 10	< 1	2
12	5	< 10	< 10	< 10	< 1	< 2

Table 5. *Serological activity and stability of immunoglobulin fractions eluted from agglutinated RBPT antigen*

Sample	SAT	Coombs test	DTT	Rivanol test	Heat* inactivation test	QRBPT	CFT
BS 1†	< 10	160	< 10	< 10	< 10	8	4
BS 2†	10	640	10	< 10	10	32	20
BS 2 (DTT reduced)	< 10	320	< 10	< 10	< 10	8	< 2
BS 2 (rivanol precipitated)	< 10	320	< 10	< 10	< 10	8	4
BS 2 (heated*)	10	320	< 10	< 10	10	32	10
BS 2 (papain digested‡)	< 10	< 10	< 10	< 10	< 10	< 1	ND

* SAT on serum heated at 65° C. for 15 min.

† Proportions of total IgG_1 globulin absorbable with *Br. abortus* cells were 17% for BS 1 and 23% for BS 2.

‡ Incubated at 37° C. for 1 hr. with 10 mg. per ml. papain + 0·001 M EDTA + 0·01 M DTT at pH 7·0.

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Table 6. *Effect of selective removal of immunoglobulin classes on serological activity of bovine antisera to* Br. abortus

Sample	SAT	Coombs	DTT	QRBPT	CFT
IBS 1* + normal rabbit serum	2560	> 10,240	2560	1024	4000
IBS 1* + rabbit anti-bovine IgM	1280	> 10,240	640	1024	1000
IBS 1* + rabbit anti-bovine IgG_1	40	640	20	< 1	10
IBS 1* + rabbit anti-bovine IgG_2	640	2560	640	512	4000
IBS 1* + rabbit anti-bovine colostrum serum (IgG_1 absorbed)	640	2560	320	256	4000

* IBS 1 = high titre serum from naturally infected cow.

Combination of immunoelectrophoresis and immunodiffusion showed that the eluates contained a single precipitating component giving a reaction of identity with bovine IgG_1 globulin (Pl. 2; Fig. 1). Diffusion of eluate against antiserum to bovine IgG_1 globulins produced a reaction of identity with a bovine IgG_1 preparation (Pl. 2; Figure 2). The proportion of IgG_1 immunoglobulin in the eluate possessing specific antibody activity was determined by radial immunodiffusion titration of samples before and after absorption with RBPT antigen. As shown in Table 5, only a minor proportion of eluted immunoglobulin had antibody activity. Presumably most antibody activity was destroyed by denaturation under the conditions of elution.

Specific immunoglobulin inhibition tests

Treatment of sera with specific antisera to bovine IgM or IgG_2 had only a marginal effect on the quantitative RBPT activity, although other serological activities were reduced. Treatment with antiserum to IgG_1, with resultant removal of detectable IgG_1 from the serum, eliminated all quantitative RBPT activity (Table 6).

DISCUSSION

Although the non-specific agglutinins of bovine serum have been at least partially characterized as a heterogeneous group of fast sedimenting proteins (Rose, Roepke & Briggs, 1964), little direct information has been presented in relation to the nature of the specific agglutinins detected by the acid plate agglutination test or its modification – the RBPT.

It is clear from previous reports that both 7s and 19s immunoglobulins may be active in the SA test (Rose & Roepke, 1964; Tailyour & Cochrane, 1966). The results of the present gel filtration and ultracentrifugation experiments confirmed this and also showed that the 19s fraction had no activity in the RBPT. The density gradient ultracentrifugation results showed coincidence of maximum quantitative RBPT titre and 7s γ-globulin concentration. This was clearly confirmed by the gel filtration results in which all RBPT activity was confined to fractions corresponding to the second absorption peak. Further substantiation was given by the results of inactivation experiments. Treatments which destroy IgM activity, such as rivanol precipitation and reduction with thiols, left the quantitative RBPT titres virtually unaffected, although reducing the SA titre

of the serum. These results suggested that RBPT activity was a property of the 7S immunoglobulin fraction.

The presence of CF, Coombs and DTT-stable agglutinating activity in the same fractions also suggested that all or some of these properties might be activities of the same antibody molecules. This possibility was examined by further fractionation experiments.

Preparative zone electrophoresis showed that agglutinins active in the SA test were present in all fractions of γ and slow β mobility. However, RBPT and CF activities were confined to the γ fractions of high mobility. The slow γ fractions only contained reduction-stable agglutinins and Coombs antibodies. These results showed that the RBPT activity was confined to a subclass of the 7S immunoglobulins. Bovine serum has been shown to contain at least three classes of 7S immunoglobulins, IgG_1, IgG_2 and IgA, as well as the 19S IgM (Murphy, Osebold & Aalund, 1965; Aalund, 1968; Mach, Pahud & Isliker, 1969; Porter & Noakes, 1970). It seemed probable that the RBPT activity was restricted to a single class of 7S, electrophoretically fast γ globulin. In an attempt to resolve this, ion-exchange chromatography on the strong anion-exchange resin QAE-Sephadex A 50 was performed. This process fractionated the γ-globulins into distinct classes. IgG_2 globulin was eluted free of other serum proteins. This did not contain RBPT or CF activity but showed DTT-stable agglutinating and Coombs antibody activities. Fractions subsequently eluted were not homogeneous, but the only ones active in the RBPT were those containing IgG_1. These fractions also accounted for most of the CF activity recovered. This suggested that the immunoglobulin class responsible for RBPT activity was IgG_1.

This conclusion was strongly supported by the results of specific-inhibition tests. Clearly IgG_1 was essential for RBPT activity and its specific removal resulted in loss of activity. Removal of IgG_2 or IgM by specific antiserum had little effect on quantitative RBPT titres.

The immuno adsorption-elution experiments confirmed the results of the previous experiments. The only serum protein detected in the eluates had the immunochemical properties of IgG_1. Its serological activity, although much reduced relative to the original serum, strongly suggested that CF and RBPT activities are properties of the same antibody molecule.

It is also apparent from the results presented that RBPT and CF activities are not necessarily equivalent to reduction-stable agglutinating and Coombs activities.

These findings are significant from the diagnostic point of view. Thus, in surveys of large numbers of sera conducted at this laboratory, Morgan *et al.* (1969) and Davies (1971) found a high degree of consistency between the results of the CF test and those of the RBPT. In some cases a positive RBPT reaction was observed in the absence of detectable CF antibody but the reverse situation was rarely encountered. These observations are explicable on the basis of the greater sensitivity of the RBPT. In the present work tests on antibodies eluted from RBPT antigen showed that these reacted to a higher dilution in the quantitative RBPT than in the CF test.

At present the CF test is widely used for differentiating cattle with persisting agglutinins resulting from *Br. abortus* Strain 19 vaccination from those naturally infected with *Br. abortus*. The selection of this procedure is based on the observations that in vaccinated cattle persisting agglutinins are likely to be due to IgM antibodies, whereas in chronically infected animals they are predominantly of the IgG type and are associated with CF activity (Yuskovets, 1956; Anderson, Jenness, Brumfield & Gough, 1964; Jenness, Anderson & Gough, 1965; Schimmel & Erler, 1967). The results of the present work suggest that a quantitative adaptation of the RBPT might offer an alternative to the CF test for detecting IgG_1 antibodies. However, it should be noted that complete correlation between the results of RBPT and CF tests should not be expected. As shown by Corbel (1972) the RBPT detects only antibodies directed against the agglutinogens of *Br. abortus*, whereas the CF test may detect antibodies to both agglutinogenic and non-agglutinogenic antigens.

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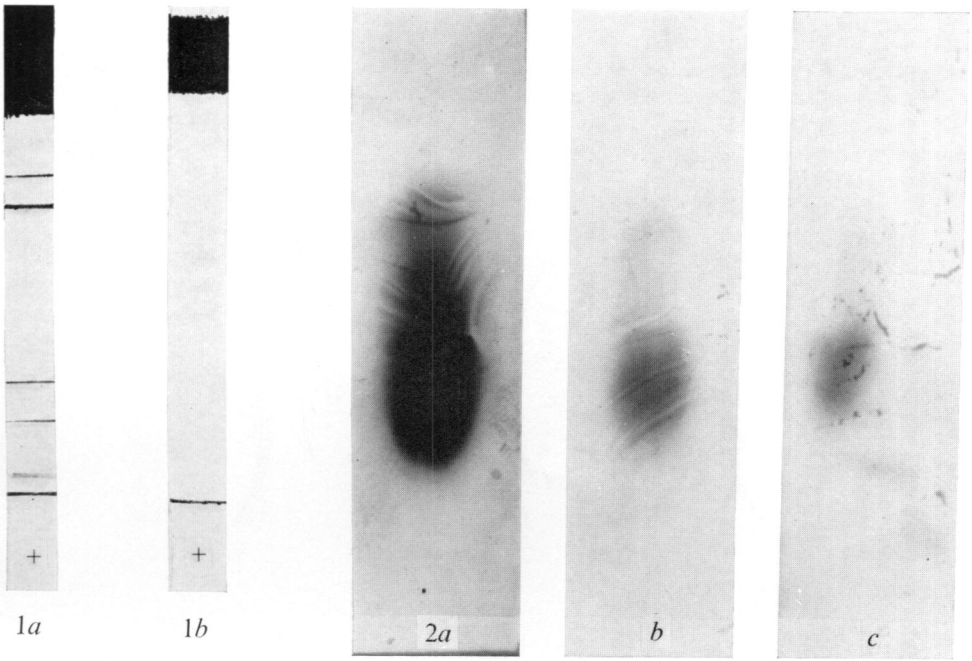
EXPLANATION OF PLATES

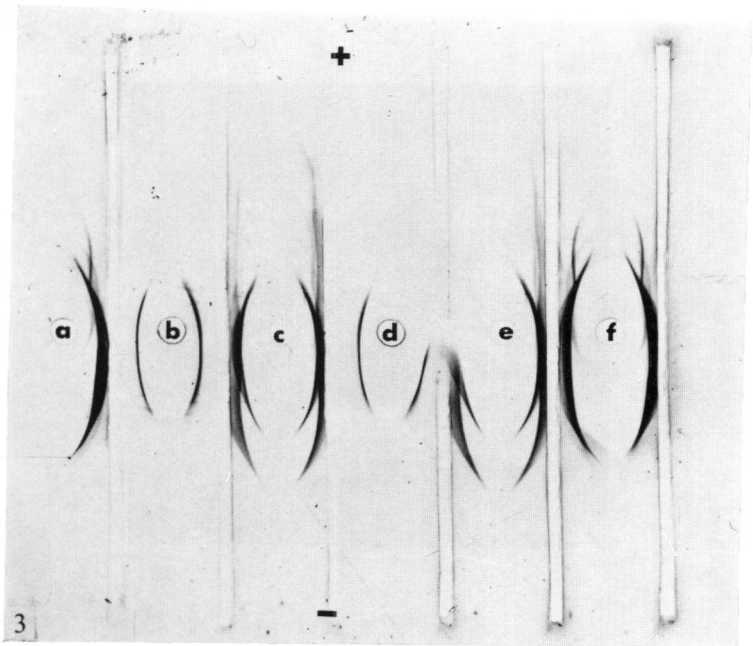
PLATE 1

Fig. 1. (*a*) Disk electrophoresis of the immunoglobulin fraction eluted from agglutinated RBPT antigen at pH 1·0. A single broad zone with an electrophoretic mobility identical with that of γ-globulin is apparent. Minor protein components, probably extracted from the *Br. abortus* cells, are also visible. (*b*) Disk electrophoresis of purified bovine IgG immunoglobulins. A single broad zone of low mobility is visible.

Fig. 2. Thin-layer gel filtration of (*a*) bovine serum, (*b*) fraction eluted from agglutinated RBPT antigen at pH 1·0, (*c*) purified bovine IgG immunoglobulins. Fraction (*b*) clearly contained components of molecular dimensions similar to IgG immunoglobulin.

Fig. 3. Immunoelectrophoresis of: (*a*) bovine γ-globulins; (*b*) fraction eluted at pH 1·0 from agglutinated RBPT antigens; (*c*) bovine serum; (*d*) and (*e*) interrupted trough method to show cross-reaction of eluted immunoglobulin fraction (*d*) with IgG_1 immunoglobulin in bovine γ-globulin fraction (*e*); (*f*) bovine γ-globulins. The antiserum used was rabbit anti-bovine γ-globulin serum.





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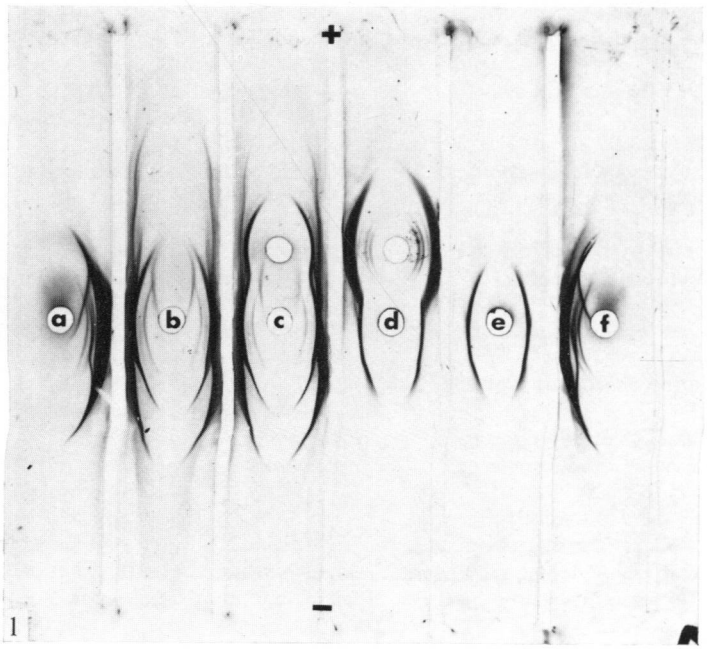

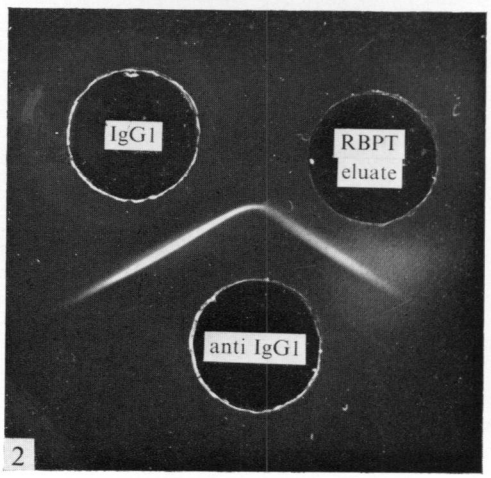

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PLATE 2

Fig. 1. Immunoelectrophoresis of: (*a*) bovine γ-globulins; (*b*) bovine serum; (*c*) and (*d*) combined immunoelectrophoresis and immunodiffusion. The upper well of (*c*) contained immunoglobulin eluted from agglutinated RBPT antigen at pH 1·0 and the lower well bovine γ-globulins. In (*d*) the positions of these reactants was reversed. In each case the eluted immunoglobulin has given a reaction of identity with the IgG_1 immunoglobulin arc. (*e*) Immunoglobulin eluted at pH 1·0 from agglutinated RBPT antigen, (*f*) bovine γ-globulins.

Fig. 2. Diffusion of immunoglobulin fraction eluted from agglutinated RBPT antigen and purified IgG_1 immunoglobulin against rabbit antiserum to bovine IgG_1 globulin. A reaction of identity was given by the two fractions.

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