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The immune response to viruses in calves

I. Response to Murray Valley encephalitis virus

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INTRODUCTION

Although the sequence of physically different antibodies in the immune response has attracted considerable attention, only relatively few animal-antigen systems have been subjected to detailed study. Uhr & Finkelstein (1967) have reviewed the work of Uhr and co-workers on the response to bacteriophage in guinea-pigs. Svehag & Mandel (1964*a*, *b*) characterized antibodies following inoculation of poliovirus in rabbits. Nossal, Austin & Ada (1965) analysed the response to *Salmonella* flagella antigen in rats. There has been very little significant work on the response in cattle, except for the study of the response to *Anaplasma marginale* by Murphy, Osebold & Aalund (1966). The differences between the responses in these systems indicates the importance of further detailed studies of a variety of animal-antigen systems.

The primary response to arboviruses in man shows the rapid development of neutralizing and haemagglutination-inhibiting (HI) antibodies; haemolytic complement fixing (HCFT) antibodies appear later and are usually relatively short lived (Southam & Green, 1958; Buescher *et al.* 1959; Scherer *et al.* 1959). In the secondary response there is a rapid increase of antibodies detected by all three serological tests and reinfection with a different but related arbovirus causes a broadly cross-reacting response in which antibodies are detected to other viruses which would not be detected in the primary response to either infecting virus (Theiler & Casals, 1958; Porterfield, 1962; Pond *et al.* 1967).

Bovine immunoglobulins have not been fully characterized although there is evidence for an immunoglobulin in addition to IgG and IgM which may be a bovine IgA (Murphy, Osebold & Aalund, 1965; Pierce & Feinstein, 1965; Winter, 1966). This paper describes the response in calves following experimental inoculation of Murray Valley encephalitis virus (MVE) using different serological tests and techniques of protein separation to study the part played by physically different immunoglobulins.

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MATERIALS AND METHODS

Serology

The HI test was carried out by the method of Clarke & Casals (1958), using kaolin adsorption to remove non-specific inhibitors (NSI). A modified direct HCFT as described by Boulanger & Bannister (1960) was used. This test will be described in more detail elsewhere (Sanderson, to be published). The plaque reduction (PR) test was carried out on the PS cell line (Westaway, 1966). This technique and some of the difficulties encountered with the assay of bovine antibody to MVE will be reported elsewhere (Sanderson, to be published).

Antibody separation

(i) Zone centrifugation was carried out by the method of Kunkel (1960). Sucrose gradients from 40 % to 10 % were produced in a gradient device (Britten & Roberts, 1960). This technique gave a complete and reproducible separation of IgM, IgG and NSI (Sanderson, 1968*b*). The amounts of IgG and IgM are expressed as the total HI units in the appropriate fractions when twenty fractions were collected.

(ii) Gel filtration in a $45 \times 3{\cdot}5$ cm. column of Sephadex G200 (Pharmacia, Uppsala, Sweden) equilibrated in 1·0 M-NaCl in 0·02 M phosphate buffer, pH 8·0, was used to separate the proteins in 3 ml. of serum. The exclusion peak contained the IgM, the natural agglutinins and the NSI (Sanderson, 1968*b*).

(iii) Anion-exchange chromatography was carried out on DEAE Sephadex A50 (Pharmacia). A 0·02 M pH 8·0 phosphate buffer was used to separate IgG into slow (IgG_S) and fast (IgG_F) fractions. Fractions containing IgG freed of IgM and NSI by gel filtration were dialysed against starting buffer and passed through a 10×2 cm. column of DEAE Sephadex. The fall through peak (IgG_S) was collected. The adsorbed protein containing IgG_F was eluted with 0·35 M phosphate buffer pH 8·0. These fractions were concentrated by dialysis against polyetheleneglycol (carbowax MW = 20,000) and then dialysed against phosphate buffered saline (PBS). They were then made up to the original serum volume (3 ml.). If the adsorbed proteins were eluted with a linear gradient from 0·02 M to 0·35 M phosphate buffer two peaks were resolved. Plate 1*a* shows the analysis of these peaks by disk electrophoresis. Peaks 1 and 2 show the separation of IgG into slow and fast fractions. Immunoelectrophoresis also demonstrated this separation.

(iv) Disk electrophoresis was used analytically as in Plate 1*a* or as a small-scale preparative electrophoretic technique to examine the properties of the antibody activity. This technique on acrylamide gel was essentially that of Davis (1964), with the following minor modifications. Gels were formed in glass tubes of 10 mm. internal diameter, 120 mm. in length. A 1 cm. spacer gel of 3·5 % acrylamide was formed over a 10 cm. separation gel of 7 % acrylamide. The tris-glycine pH 8·9 electrode buffer and tris-HCl gel buffer of Davis were used. A few drops of concentrated bromophenol blue were placed in the upper electrode chamber. The density of a 0·4 ml. sample was increased by adding 10 % sucrose and the sample layered on the gel under the electrode buffer. A current of 5 mA. per tube was applied until the sample had entered the spacer gel; the current was then increased

to 10 mA. per tube until the bromophenol blue had reached the bottom of the tube gel. For antibody assay the gels were removed and sliced transversely (the spacer gel was not sliced) into disks weighing approximately 0·2 g. These were macerated with 0·4 ml. of PBS and allowed to stand overnight before titrating the supernatant for antibody activity. Alternatively gels were stained as described by Davis, although in this case the serum or serum fractions were diluted 1/20 before applying to the gel.

Experimental animals

Calves were purchased at local sales. They were of mixed dairy breeding and had been raised close to Brisbane, but no further details on their history were available. Their ages were estimated by physical appearance and so can only be regarded as approximate: calf 95, aged 5 months; 96, aged 5 months; 97, aged 3 months; 98, aged 3 months. The calves were maintained in insect-free housing.

Each calf was given a subcutaneous injection of 3 ml. of MVE infected unweaned mouse brain homogenate (containing approximately 10^8 LD50/0·1 ml. in unweaned mice). Calves 96 and 98 were given a second dose of the same material 34 days after the first to elicit a secondary response. Details of the virus strains have been given elsewhere (Sanderson, 1968*b*). Another group B arbovirus, strain MRM 3929 (R. L. Doherty, personal communication) was used in the fourth unweaned-mouse brain passage.

RESULTS

Calf 95

Antibody detected by the HI test approximately paralleled antibody detected by the PR test (Fig. 1). The HCFT antibody appeared only slightly later than HI antibody and reached maximum titre by the 8th day. Examination of the samples after zone centrifugation showed that up to the 10th day IgM was the major antibody present. After this it decreased and was not detectable by the 18th day. IgG was present, although in lesser amounts, as early as the 7th day and by the 15th day it was present in greater amounts. The HCFT antibody paralleled the IgG. Relatively little IgG_S was formed; it reached a maximum titre of 8, whereas the IgG_F reached a maximum titre of 160. The HCFT titre was less than 2 in the IgG_S fractions.

Calf 96

This calf (Fig. 2) showed a similar response to calf 95 as detected by the HI, HCFT, and PR tests. The second injection of MVE caused a drop in HI titre which reached its lowest level 2 days after the injection. This was followed by a classic secondary response with an increase in antibody detected by all three tests. Maximum HI and HCFT titre was reached by the 6th day but by the 10th day it was declining rapidly. After the 13th day samples were taken at 1 month intervals for 12 months. There was no significant fall in HCFT titre relative to HI titre; antibody detected by all tests fell to a very low level rapidly. Antibody was detectable by HI for only 8 months.

Zone centrifugal analysis showed that the first antibody detectable was IgG, and although a distinct IgM peak occurred, the IgG was at all times present at

higher titre than IgM. Although it is difficult to compare the responses in the two calves because of the limited number of samples collected at this early stage of the response, it appeared that the IgG in calf 96 reached detectable levels earlier than

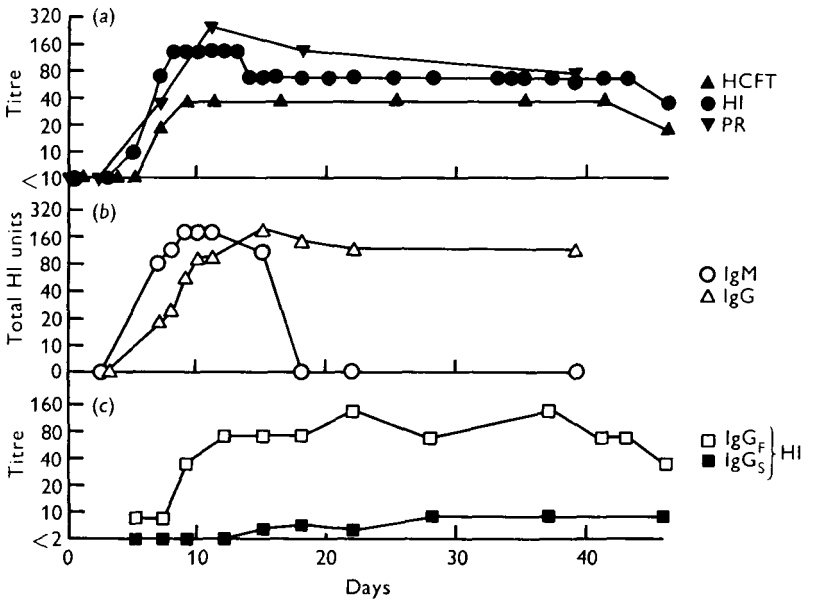

Fig. 1. Response in calf 95. (*a*) Total antibody response (whole serum). (*b*) Total HI antibody in IgM and IgG sucrose gradient fractions. (*c*) Antibody in IgG_F and IgG_S after separation on DEAE Sephadex.

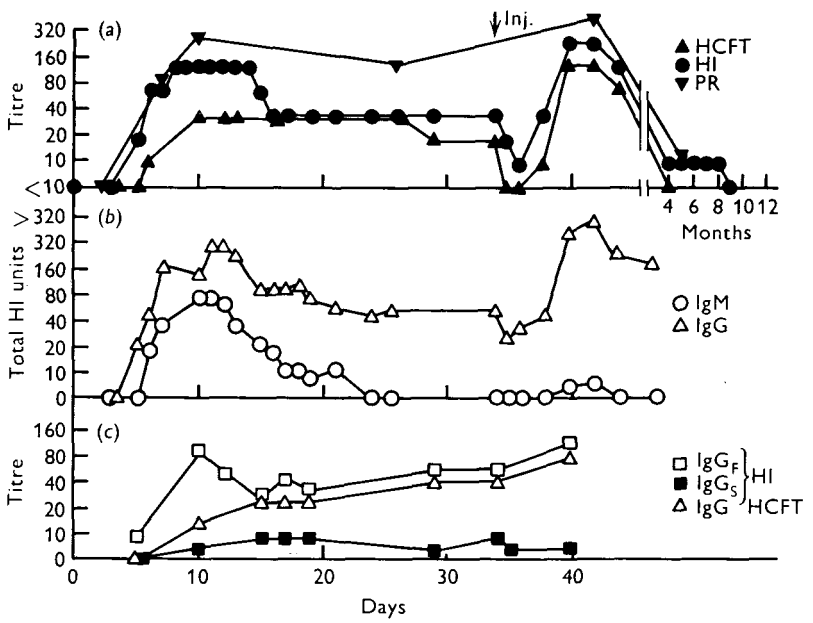

Fig. 2. Response in calf 96. (*a*), (*b*) and (*c*) as in Fig. 2.

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in calf 95 and reached a higher titre. On the other hand, the IgM in calf 96 did not reach the same peak titre as in calf 95 although it was detectable for a longer period. In the secondary response calf 96 formed very little IgM; at its highest point only a total of six HI units were detected in the sucrose gradient.

Most of the HI and all of the HCFT antibody in both the primary and the secondary response was IgG_F. The IgG_S reached a maximum HI titre of 8, but the HCFT titre was at all times less than 2 (there was no anti-complementary activity in these fractions).

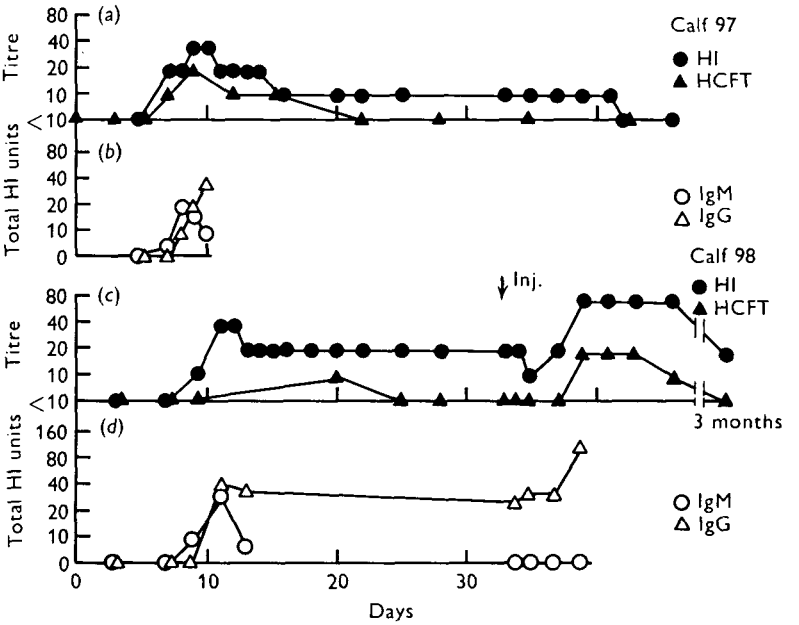

Fig. 3. (*a*) Total antibody response (whole serum) in calf 97. (*b*) IgM and IgG response in calf 97. (*c*) Total antibody response (whole serum) in calf 98. (*d*) IgM and IgG in calf 98.

Calf 97

The response in this animal (Fig. 3) was lower in titre than in calves 95 or 96. HCFT antibody was detected on the same day as the first HI antibody. A low titre of IgM was detected on the 7th day, and on the 8th day IgG had also appeared. Serum samples taken 12 and 28 days after inoculation contained only IgG_F antibody activity.

Calf 98

This animal (Fig. 3) showed a similar low-level response. An HI titre of 20 was maintained until the 34th day when a second injection was given. After this the HI titre fell slightly on the 2nd day, and then increased by the 4th day, reaching a maximum titre of 80 by the 6th day. Only a low level of HCFT antibody was detectable in the primary response; it appeared about 6 days after the second inoculation. IgM was first detected on the 9th day and reached a maximum about the 11th day and decreased rapidly. No IgG was detected on the 9th day but it had

reached a maximum titre by the 11th day. No IgM was detected in the secondary response. Serum samples 12 and 28 days after the first inoculation contained only IgG_F.

IgM complement-fixing antibody

Sucrose gradient fractions from calves 95 and 96 containing relatively high levels of IgM antibody were dialysed against PBS, concentrated and tested by HI and HCFT. Several samples with HI titre from 32 to 128 were tested but none showed any complement fixation, although they had an anti-complementary titre of 4 to 16 which may have masked a low level of fixation. It was clear from these tests that the HCFT was at least 16-fold less sensitive than the HI test in detecting IgM.

Electrophoretic properties of IgG_F

To examine the electrophoretic nature of the material regarded as IgG_F, fractions were titrated after electrophoresis. All the serum samples separated by anion exchange chromatography from calves 95 and 96 and the samples at 12 and 28 days from calves 97 and 98 were examined, and within the limitations of the technique there was no indication of heterogeneity. The distribution of both HI and HCFT antibody was at all times similar to the distribution of HI antibody shown in Plate 1*b*.

Table 1. *Cross-reactions in MVE antiserum*

	Days after injection	HI titre* MRM 3929	Kunjin	MVE	Type of antibody
Calf 95	7	20	40	80	Whole serum (IgM)
	10, 15	40	20	160	Pooled sucrose gradient (IgG)
	42	40	20	160	Whole serum (IgG)
Calf 96	5	< 20	< 20	40	Whole serum (IgG)
	10	80	40	160	Whole serum (IgG and IgM)
	10	40	< 10	80	Heated whole serum (IgG)
	10, 11	< 20	40	80	Pooled sucrose gradient (IgM)
	42	320	80	1280	Whole serum (IgG)

* Samples early in the response were also tested for reactions to Kokobera, Stratford and Edge Hill. They were all less than 20, and are therefore not listed.

Cross-reactions

These are shown in Table 1. In calf 95 the first antibody formed was IgM when whole serum showed cross-reactions (HI) with Kunjin and MRM 3929 viruses, but not with Kokobera, Stratford or Edge Hill. Heating at 64° C. for 30 min. destroyed the HI activity, confirming that the reactions were due to IgM (Sanderson, 1968*b*). IgG antibody pooled from sucrose gradients of samples taken 10 and 15 days after inoculation also showed cross-reactions to the same viruses although to different titres, as did a whole serum 42 days after inoculation when IgG was the only antibody present.

In calf 96 the pre-inoculation sample contained no antibody to any of these six group B viruses. The first antibody detected was IgG and 5 days after inoculation this antibody reacted only with homologous virus antigen. At 10 days whole serum

cross-reacted with both Kunjin and MRM 3929, but not with any of the other viruses. After heating this sample to inactivate IgM, the cross-reactions to Kunjin disappeared and thus the IgG reacted only with MVE and MRM 3929. Pooled IgM from sucrose gradients indicated that this immunoglobulin reacted more to Kunjin than to MRM 3929. At the peak of the secondary response when only IgG was present, whole serum cross-reacted with Kunjin but to higher titre to MRM 3929.

DISCUSSION

With the exception of calf 96 the first antibody to appear was IgM, which was rapidly followed by IgG, This explains why there was very little delay in the appearance of HCFT antibody. A clear biphasic response as has been reported in most other systems (Uhr, 1964; Svehag & Mandel, 1964*a*; Nossal *et al.* 1965) did not occur in the bovine response to MVE.

There was very little synthesis of IgM in the secondary response. The part played by IgM in the secondary response has been the subject of considerable discussion (Uhr & Finkelstein, 1967). Svehag & Mandel (1964*b*) found that the induction period, rate and duration of synthesis of IgM was similar in both the primary and the secondary response to poliovirus in the rabbit; there was no immunological memory to IgM nor inhibition of synthesis by pre-existing antibody. On the other hand, Nossal *et al.* (1965) were able to define conditions where the additional antibody in the secondary response to flagellin in rats was entirely IgM. Finkelstein & Uhr (1964) showed that passively administered antibody inhibited the synthesis of antibody, particularly IgM; this inhibition was more effective with secondary response IgG. While this appears a satisfactory explanation for the observed lack of IgM in the bovine secondary response, the secondary response in young calves described in the second paper in this series (Sanderson, 1968*a*) also showed a virtual lack of IgM, even without pre-existing primary response antibody.

The primary response in calf 96 was unusual in that IgG was detected before IgM. Such a possibility was suggested by Schoenberg *et al.* (1965) in contrast to the claim by Nossal *et al.* (1964) that cells first form IgM and then switch to IgG synthesis. However, the possibility must be considered that the response in calf 96 was not a true primary response but influenced by a previous natural arbovirus infection. It would seem unlikely that it represented a secondary response to homologous virus because there was no detectable antibody in the pre-inoculation sample, and the response did not resemble the experimental secondary responses, which showed a virtual absence of IgM and different kinetics of antibody formation. The response may be secondary, following natural infection by a different but related virus; thus the earliest IgG may represent a secondary response to shared specificity, and the IgM and some of the later IgG a primary response specific for MVE.

This type of reaction has been studied in relation to antigenically related proteins (Dixon & Maurer, 1955; Wiegle, 1961; Gilden & Tokuda, 1963). It is also a well-known phenomenon with influenza viruses where vaccination with a new

strain of virus results in antibody directed mainly towards the first strain encountered (Davenport & Hennessy, 1956; Webster, 1966). After reinfection with a related group B arbovirus, antibodies are formed which cross-react broadly within the group (see Introduction). In calf 96 there was no antibody in the pre-inoculation sample to any of the six group B arboviruses tested. Cross-reactions occurred to MRM 3929 and Kunjin in the early IgG, but to no greater extent than occurred in calf 95 (Table 1). No cross-reactions were detected to Stratford, Edge Hill or Kokobera at any stage of the response to the first inoculation of MVE. This suggests that it was not influenced by previous arbovirus infection.

These cross-reactions are also interesting in that IgM and IgG showed different degrees of cross-reactions to other viruses. In both calves 95 and 96 the IgG reacted more to MRM 3929 than to Kunjin, and the relative titres remained similar throughout the primary and secondary response. On the other hand the IgM from both calves reacted to higher titre to Kunjin than to MRM 3929. This is further evidence that the change from IgM to IgG does not represent a total or a random switch over of a proportion of cells from IgM to IgG synthesis, as this should result in IgM and IgG of similar specificity. The fact that there is a difference in specificity emphasizes the importance of defining the immunoglobulin type in the classification of micro-organisms by their antigenic relationships.

Most of the IgG activity was due to the electrophoretically fast IgG_F; the IgG_S had only low titre HI activity and no HCFT activity. The HCFT was only about twofold less sensitive than the HI test in detecting IgG_F, whereas it was at least 16-fold less sensitive in detecting IgG_S. This suggests first that there is a difference in biological activity between the two immunoglobulins, and secondly that the antigens must been have strongly basic; that is, carry a net negative charge at physiological pH (Sela & Mozes, 1966). There is as yet very little information on the physical properties of arboviruses; however, they are eluted from DEAE cellulose only at high ionic strength at pH 8 (Nicoli, 1965), suggesting that they have low pI; that is, a net positive charge at this pH. Thus the net charge on the virus particle is not as important in determining the type of antibody formed as it is with the smaller molecules used by Sela & Mozes (1966). This could be interpreted as evidence that the virus particles are broken down, separating the positively charged part (lipid?) from the negatively charged antigen (protein?) before making contact with the factors (cells?) determining the type of immunoglobulin formed.

Following the second inoculation of MVE there was a decrease in circulating antibody titre due to the combination of virus with antibody. This did not reach its lowest level until 2 days after the inoculation. Had this been caused by the injected virus the decrease in titre should have occurred within hours, which suggests that some multiplication of virus took place in the presence of circulating antibody.

SUMMARY

Following inoculation of Murray Valley encephalitis virus into calves there was an early transient IgM response. The appearance of IgG was only slightly later than the first appearance of IgM in three calves, and in one calf IgG was detected

before IgM. The secondary response was characterized by the more rapid appearance of IgG and the virtual absence of IgM. The IgG was of the electrophoretically fast type; there was an almost insignificant amount of antibody activity in electrophoretically slow fractions. IgM and slow IgG had no complement-fixing activity. IgM and IgG showed different cross-reactions to other group B arboviruses. The different cross-reactions, and the appearance of IgG before IgM in one animal suggests that the switch-over from IgM to IgG synthesis in the response is not the result of either a total or a random proportion of cells producing IgM, changing to IgG production. The fact that the IgG was almost exclusively of fast electrophoretic mobility suggested that the virus antigenic components were strongly basic, and as this is contrary to the chromatographic properties of arboviruses, it is suggested that the virus particles must be broken down before making contact with the factors determining the type of immunoglobulin formed.

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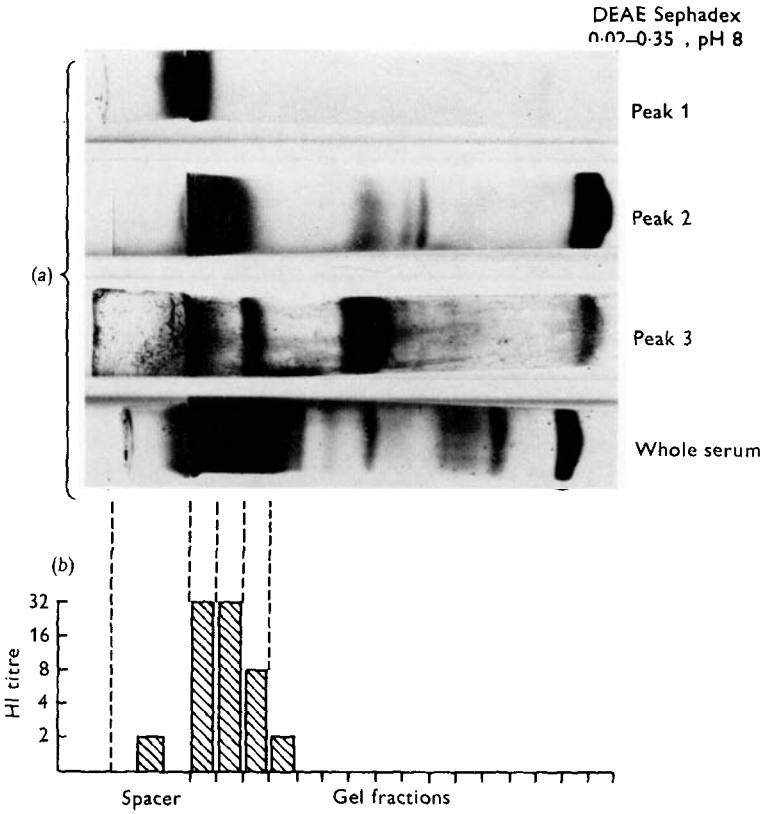
EXPLANATION OF PLATE

(*a*) Disk electrophoretic analysis of peaks 1, 2 and 3 from DEAE Sephadex chromatography, and whole bovine serum. (*b*) Titration of antibody eluted from gel segments corresponding to the stained gels of (*a*).



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