Immunology, 1965, **8**, 106.

Biophysical and Immunological Studies on Bovine Immune Globulins with Evidence for Selective Transport within the Mammary Gland from Maternal Plasma to Colostrum

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(*Received* 20*th December* 1963)

Summary. Three immune globulins in maternal serum and colostrum and newly born calf serum, have been characterized and compared. An examination was made to determine first, which of the maternal serum immune globulins accumulate in the circulation of the calf and secondly, the selectivity of the mammary gland for these proteins compared with the intestinal mucosa of the newly born calf.

By difference in their electrophoretic mobilities three antigenically related immune globulins were isolated from bovine serum.

The immune lactoglobulins in bovine colostrum were qualitatively similar to those in serum. However, marked differences were observed between the relative concentrations in serum and colostrum of the three immune globulins.

An electrophoretically fast immune globulin (C1), present in colostrum at high concentration, was shown to be antigenically similar to an immune globulin (S1) present in the maternal serum at low concentration.

These findings indicate that the mammary gland showed a highly selective preference for, and hence ability to concentrate in, colostrum, the electrophoretically fastest serum immune globulin.

The slowest serum immune globulin and the component with intermediate electrophoretic mobility (S3 and S2 respectively) were both present at high concentration in bovine maternal serum, but were transmitted at different rates into the colostrum, so that the slowest serum immune globulin (S3) was present in the colostrum as a comparatively minor component (C3).

In contrast to the mammary gland, the intestine of the newly born calf (permeable to undegraded protein during the first 24 hours of life) showed no selectivity. Immune globulins showing the three electrophoretic mobilities were absorbed equally readily.

Thus, while the bovine mammary gland showed a highly selective preference for certain electrophoretically different serum proteins, no comparable selectivity was shown by the intestinal mucosa of the newly born calf.

The results emphasize the heterogeneity of bovine immune globulins and show that the calf receives into its circulation from ingested colostrum selected maternal serum immune globulins. This selection of proteins from maternal plasma, for admission to the calf's circulation, occurs within the mammary gland during the formation of colostrum but not during absorption across the calf's intestinal mucosa.

INTRODUCTION

Evidence is accumulating to show that the immune lactoglobulin in colostrum is derived directly from the maternal serum immune globulin without obvious degradation

and resynthesis (Askonas, Campbell, Humphrey and Work, 1954; Larson and Gillespie, 1957; Larson, 1958; Dixon, Weigle and Vazquez, 1961; Feldman, 1961).

In man more extensive investigations have been carried out, and an immunological relationship has been established between the immune globulins in serum and the immune lactoglobulins in milk (Gugler, Bokelmann, Dätwyler and von Muralt, 1958; Hanson, 1960, 1961; Huidobro-Tech, 1959; Karte, 1959; Lunsford and Deutsch, 1957; Schwick, Esser and Koch, 1959; Da Silva and Monteiro, 1959). For some species a selectivity has been shown in the absorption of immune globulins across certain biological membranes. For example, homologous γ-globulin is more readily transmitted than is heterologous across the yolk-sac splanchnopleur to the rabbit embryo (Brambell, Hemmings, Henderson and Rowlands, 1950; Batty, Brambell, Hemmings and Oakley, 1954; Hemmings, 1956, 1957; Hemmings and Oakley, 1957). A similar selectivity occurs from the yolk-sac to the chick (Brierley and Hemmings, 1956) and across the intestinal epithelium to the newly born rat (Halliday, 1955, 1958).

Hartley (1948, 1951) showed that homologous diphtheria antitoxin was transmitted from the mother to the guinea-pig foetus more readily than equine antitoxin. Pepsin-refined antitoxin, whether derived from homologous or heterologous serum and having approximately half the molecular weight of the original molecule, was very poorly transmitted. Similarly in the rabbit, natural homologous antitoxin was transmitted to the foetal circulation more readily than pepsin-digested antitoxin (Brambell, Hemmings and Oakley, 1959). These results were confirmed using papain digest fragments of homologous γ-globulin. Porter fraction III was transmitted nearly as readily as whole γ-globulin, whereas fractions I and II were transmitted less readily (Brambell, Hemmings, Oakley and Porter, 1960).

The selective transport of serum γ-globulin, compared with other homologous serum proteins, has been shown for the placenta of the rhesus monkey (Bangham, Hobbs and Terry, 1958; Bangham, 1960) and also across the secretory epithelium of the follicle into the developing ova of the fowl (Patterson, Youngner, Weigle and Dixon, 1962).

In any species where the different immune globulin components can be differentiated by their electrophoretic behaviour, differences could exist between the rate of transport of the various immune globulin components across biological membranes. This could account for a similarity between the electrophoretically faster serum immune globulins and the immune lactoglobulins in bovine colostrum (Smith, 1946; Pierce, 1955). Larson (1958) also adduced evidence, on the basis of electrophoretic mobility, for the selective concentration of γ_1 and β_2 rather than γ_2 plasma proteins in bovine colostrum.

The present study identifies and examines quantitatively the various immune globulin components recognizable in bovine serum and colostrum using critical fractionation procedures. An immunochemical and physical comparison is made between the immune globulin components identified in bovine serum and colostrum and also between those which are absorbed from the intestine and which accumulate passively in the serum of the newly born calf after suckling.

MATERIALS AND METHODS

Collection and Preparation of Bovine Sera and Colostrum

The experiments were carried out on serum derived from a Jersey cow (E4) and a colostrum sample from the same cow at calving. Several steers, about 1 year old, provided

blood from which a pooled serum sample was prepared. Whole colostrum was clarified by centrifugation at ~18,000 *g* for 30 minutes.

Preparation of Antisera

Rabbits were repeatedly immunized by several courses of graded doses of antigen given intravenously or intramuscularly. Blood was drawn from the ear veins and, after standing overnight at room temperature, serum was separated from the clot by centrifugation. The colostrum and sera were stored at $-10°$ or $-20°$.

FRACTIONATION METHODS

(a) *Starch grain electrophoresis* was carried out using the technique of Paigen (1956). The starch block, measuring $27 \times 19{\cdot}5 \times 1{\cdot}2$ cm., and bovine serum were separately equilibrated with phosphate buffer, pH 8, $I = 0{\cdot}1$. A trough 1 cm. wide, cut across the middle of the block, was packed with a starch paste containing approximately 10 ml. of serum. After the electrophoresis of the proteins, sections 1 cm. wide and parallel to the starting zone were removed and eluted with phosphate buffer through a sintered glass funnel.

(b) *Sephadex.* Serum protein fractions obtained by electrophoretic separation, were further fractionated in phosphate buffer (pH 8, $I = 0{\cdot}1$) through a Sephadex G200 column. The eluate was collected in a fraction collector and E at 280 mμ measured using a Unicam spectrophotometer S.P.500.

(c) *Chromatography.* Bovine colostrum was fractionated on a diethylaminoethylcellulose (DEAE-cellulose) column using the phosphate buffer system with gradient elution described by Fahey, McCoy and Goulian (1958). The elution diagram was plotted as for Sephadex fractions. The concentrations of protein in large volumes of solute were raised by pervaporation; for smaller volumes, osmotic concentration was carried out using carbowax.

IMMUNOLOGICAL METHODS

(a) *Agar-gel precipitin tests.* The double-diffusion precipitin method of Ouchterlony (1949, 1958) was used to establish immunological relationships between various fractions of serum and colostral protein. Ion agar was used at 1 per cent (w/v) in phosphate buffer, pH 7·4, containing 0·15 M NaCl and 0·1 per cent (w/v) sodium azide. The sizes and positions of the wells were varied according to the characteristics of the reactants and the amount of material available.

(b) *Immunoelectrophoresis.* A modification of the technique of Grabar and Burtin (1960) was employed, using 1·5 per cent (w/v) agar gel containing 0·1 per cent (w/v) sodium azide and veronal buffer, pH 8·6, $I = 0{\cdot}025$. A field strength of 7 volts/cm. for 2 hours was applied across the agar. In some instances the identity of certain precipitin arcs was confirmed by the diffusion of an appropriately purified and identified protein fraction from a second well, cut parallel to the antiserum well, but on the opposite side of the electrophoretically dispersed antigen. A line of precipitate develops parallel to the two troughs and fuses with that arc due to a similar antigenic component in the immunoelectrophoretic pattern (Osserman, 1960).

Starch gel electrophoresis was sometimes used as a method of fractionation of bovine immune globulins prior to examination by immunoelectrophoresis. Part of the starch gel was stained for protein and, from an adjacent portion, pieces of starch were removed

and fitted into congruent holes cut in agar gel; the proteins were moved electrophoretically from the starch into the agar and were then examined immunoelectrophoretically (see Figs. 3 and 4).

(c) *Starch gel immunoelectrophoresis.* Occasionally, after electrophoresis in starch gel, components were located by allowing the protein to diffuse out of the starch into agar and towards an appropriate antiserum in a trough cut in the agar parallel to the starch gel inlay (see Fig. 2).

ANALYTICAL METHODS

(a) *Starch gel electrophoresis* followed, with slight modifications, the technique developed by Smithies (1955); phosphate buffer, pH 7·6, was used (Ashton, 1957). A current density of approximately 5 mA./cm.^{2} cross-section was applied for 4 hours. Protein bands were located using Naphthalene Black 10 B.

(b) *Ultracentrifugation.* The Svedberg and Spinco model E analytical ultracentrifuges were used. Runs on the Svedberg ultracentrifuge were kindly carried out by Dr. R. A. Kekwick of the Lister Institute, London, and runs using the Spinco ultracentrifuge by Dr. P. Johnson, Department of Colloid Chemistry, Cambridge.

(c) *Chromoscan analyses.* Estimations were made from planimetric analyses of Chromoscan patterns of photographs of proteins separated by starch gel electrophoresis and stained with Naphthalene Black.

Nomenclature. In the absence of any well-established nomenclature for bovine serum immune globulins and immune lactoglobulins, based on antigenic relationships, the components in which the various immune globulins were located, after separation by starch gel electrophoresis, were designated S1, S2 and S3 for serum and C1, C2 and C3 for colostrum, in order of descending mobility. In general terms the mobility of S1 and C1, S2 and C2, and S3 and C3 components are similar.

RESULTS

STARCH GEL ELECTROPHORESIS

Adult bovine serum examined by starch gel electrophoresis showed a broad component on the cathode side of the insert which is characteristic of the serum immune globulins (Fig. 1e). Sometimes this component could be resolved into two, and these components have been designated S2 and S3 (Fig. 1b). Planimetric analysis of the scanned pattern of the S2 and S3 components (Fig. 1b) showed that they were present in approximately equal proportions (S2, 47 per cent; S3, 53 per cent).

Recent work (for references see Introduction) has shown that the immune globulin present in colostrum is derived from the maternal serum with little or no degradation and resynthesis. However, although the colostrum showed a well-defined component C2, with a mobility comparable with the faster serum component S2, a component C3 with a mobility comparable with the slow serum component S3 was almost completely absent from colostrum (Fig. 1a). The examination of other colostra showed that this was a consistent trend.

Attention was directed towards a third electrophoretic component C1 (Fig. 1a), present in all the colostra examined, and moving from the origin towards the anode. A planimetric analysis of a scanned pattern for whole bovine colostrum, after electrophoretic separation of the proteins, showed that the C1 component contributed 50 per cent of the

total protein, compared with 26 per cent by the C2 component. However, the C1 component will subsequently be shown to be heterogeneous. The potential importance of the C1 component was appreciated when it was shown to be absorbed from the intestine without change in mobility, and to accumulate at the same time as the C2 component in the agammaglobulinaemic serum of the newly born calf after suckling (Fig. 1c cf. Fig. 1d).

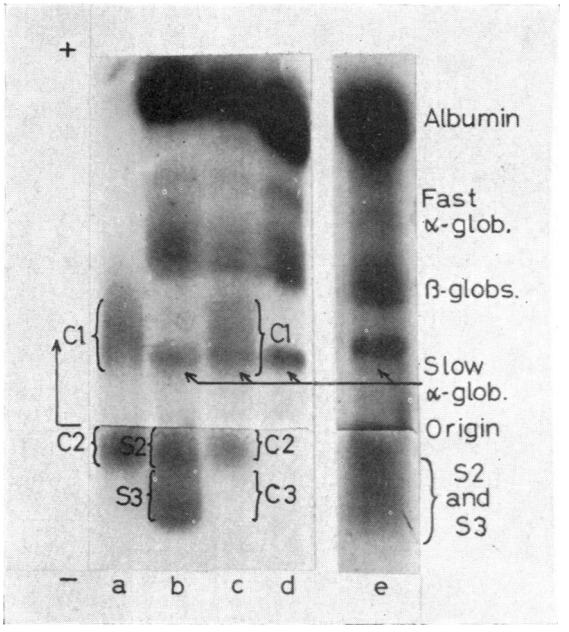

FIG. 1. Starch gel electrophoresis: (a) Bovine colostrum; (b) and (e) adult bovine serum; (c) post-colostral calf serum; (d) pre-colostral calf serum.

When the maternal (E4) serum proteins were examined no well-defined component comparable to the C1 component could be distinguished. Further experiments were carried out, first to determine whether there was any antigenic relationship between the colostral C1 component and the immune globulin present as the C2, S2 and S3 components, and secondly, to examine the bovine serum proteins more critically for evidence of proteins, in particular immune globulin, electrophoretically and antigenically resembling those present in the colostral C1 component.

PRELIMINARY STUDIES ON THE ANTIGENIC RELATIONSHIP BETWEEN THE IMMUNE GLOBULINS IN BOVINE SERUM AND COLOSTRUM

Protein was precipitated from either serum or colostrum at 12 g. Na_2SO_4 per 100 ml. The precipitates were redissolved and examined by starch gel electrophoresis. The protein precipitated from the serum contained the S2 and S3 components and that from colostrum, the C2 and C1 components (Fig. 2 a–d).

The antigenic relationship between these components was shown by starch gel immuno-electrophoresis (see Immunological methods (c)) and by double diffusion in agar gel. The binodal pattern of the immune precipitate, obtained with the colostral globulin,

showed that two distinct cross-reacting immune globulins were present as C2 and C1 components (Fig. 2 b and e). However, this fraction sedimented largely as a single peak ($s = 6{\cdot}75S$) with evidence of lighter material (Fig. 2f). The complete immunological identity of the precipitin reactions between C1, C2 and S2 and S3 diffused from starch gel cut-outs (Fig. 2g) is merely apparent. Spurs developed later, indicating only partial identity.

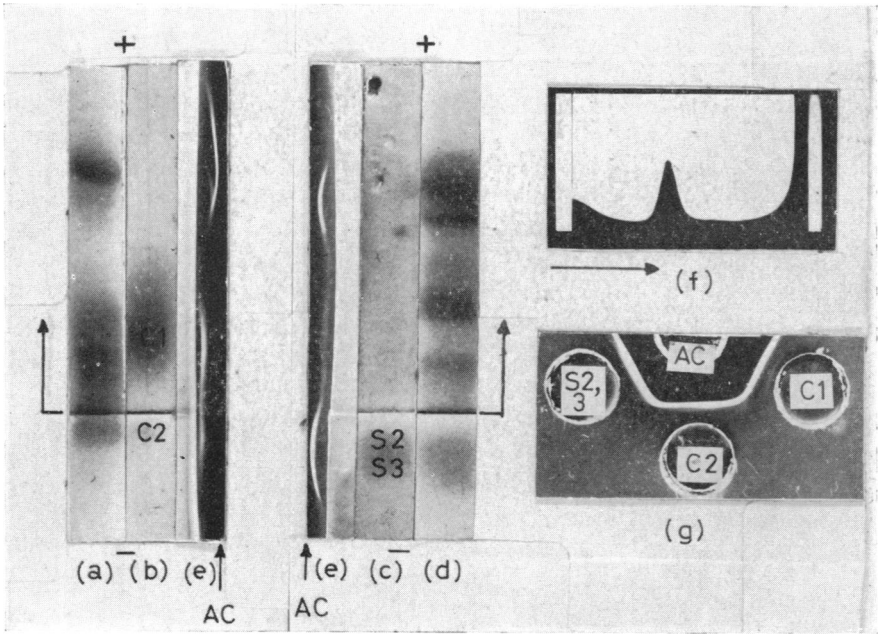

FIG. 2. Starch gel electrophoresis: (a) bovine colostral whey; (b) immune lactoglobulin precipitated at 12 g. Na_2SO_4 per 100 ml. bovine colostrum; (c) immune serum globulin precipitated at 12 g. Na_2SO_4 per 100 ml. adult bovine serum; (d) adult bovine serum; (e) starch gel immunoelectrophoresis of fractions shown at (b) and (c) against rabbit anti-bovine colostrum (AC); (f) ultracentrifuge pattern for the immune lactoglobulin fraction; (g) agar gel precipitation with cut-outs from areas marked C1 and C2, and S2 and S3 from starch gel patterns shown at (b) and (c) against rabbit anti-bovine colostrum (AC).

Bovine colostrum and serum were fractionated to isolate the various immune globulins in an attempt to characterize them and establish more precisely their immunochemical relationship.

DEAE-CELLULOSE FRACTIONATION AND IMMUNOELECTROPHORETIC ANALYSIS OF THE IMMUNE GLOBULINS IN BOVINE COLOSTRUM

Since agar immunoelectrophoresis, in addition to starch gel electrophoresis, was one of the analytical methods used for these studies, it was necessary to isolate the immune globulins identified as the C1 component by starch gel and characterize their immunoelectrophoretic behaviour in agar gel. Fractions of colostral protein were collected by gradient elution from a DEAE-cellulose column and examined by starch gel and agar immunoelectrophoresis. Discs were cut from starch gel after the electrophoretic separation of DEAE-cellulose fractions 3 and 6 (Fig. 3). The protein bands sampled were selected

after reference to an adjacent strip stained for protein, and the starch discs were inserted into holes cut in agar. The protein within the starch insert was moved electrophoretically into the agar. Subsequently rabbit anti-bovine colostrum serum (AC), was diffused into the agar from one side and Armour serum γ-globulin diffused across the agar from a parallel trough cut on the opposite side.

The results show first, that some colostral protein in the C1 component, immunologically cross-reacting with serum γ-globulin, moved towards the anode in starch as a discrete fraction—'$C1_3$' (Fig. 3a). This component extends from around the well towards the cathode in agar electrophoresis (Fig. 3d). Thus part of the immune globulins, moving towards the anode on starch gel electrophoresis, moves towards the cathode in agar gel and overlaps the mobility shown by the immune globulin in the C2 component (C2 fraction 3, Fig. 3 a and d). Secondly, immune globulin isolated at '$C1_1$' and '$C1_2$' (Fig. 3a), (from DEAE-cellulose fractions 3 and 6) showed a faster mobility in agar gel (Fig. 3d) and '$C1_1$' appeared to be responsible for the characteristically faster immune globulin (shown at 'x' in Fig. 3c) when whole colostrum is examined immunoelectrophoretically.

The immune globulin present in the C2 component (shown at C2 in fraction 3, Fig. 3a) gave a precipitin arc on immunoelectrophoresis (Fig. 3d) comparable in position with that of the faster immune globulins when whole serum was examined immunoelectrophoretically (Fig. 3c).

The appearance of the antigenically cross-reacting components $C1_1$, $C1_2$ and $C1_3$ also indicates the heterogeneity of the C1 component.

DEAE-CELLULOSE AND STARCH GRAIN FRACTIONATION, AND STARCH GEL AND IMMUNOELECTROPHORETIC ANALYSIS OF THE IMMUNE GLOBULINS IN BOVINE SERUM

Starch and Agar Gel Immunoelectrophoresis of E4 Bovine Serum

The immunoelectrophoretic pattern for bovine serum (E4) did not show a well-defined extension of the immune globulin arc towards the anode comparable with that shown by colostrum (see Fig. 3c). However, although starch gel electrophoresis failed to reveal a well-defined anodic component, comparable with the colostral C1 component, the immunoelectrophoretic pattern suggested that immune globulin corresponding to this component, or part of it, probably was present, although at lower concentration. This was confirmed by the agar immunoelectrophoretic examination of fractions, derived from the electrophoretic separation of E4 maternal serum on starch gel, which showed the presence of traces of protein cross-reacting with Armour bovine γ-globulin and associated with sections of starch removed from the anode of the insert (Fig. 4 a, b and c). However, no immune globulin could be isolated from starch gel sections on the anode side of the insert which showed, on agar immunoelectrophoresis, an arc extending towards the anode and comparable with the immune globulin from the electrophoretically fastest zone of the C1 component.

Not unexpectedly, the well-defined serum globulin component moving as a sharp band (shown at A in Fig. 4a), with an electrophoretic mobility in starch gel within the range covered by the C1 component, gave no cross-reaction with bovine γ-globulin (Fig. 4b). This component was a slow α-globulin (see Fig. 4 b and c) and can also be seen at high concentration in pre-colostral serum (see Fig. 1d).

These findings could be explained by low concentrations of the faster immune globulins

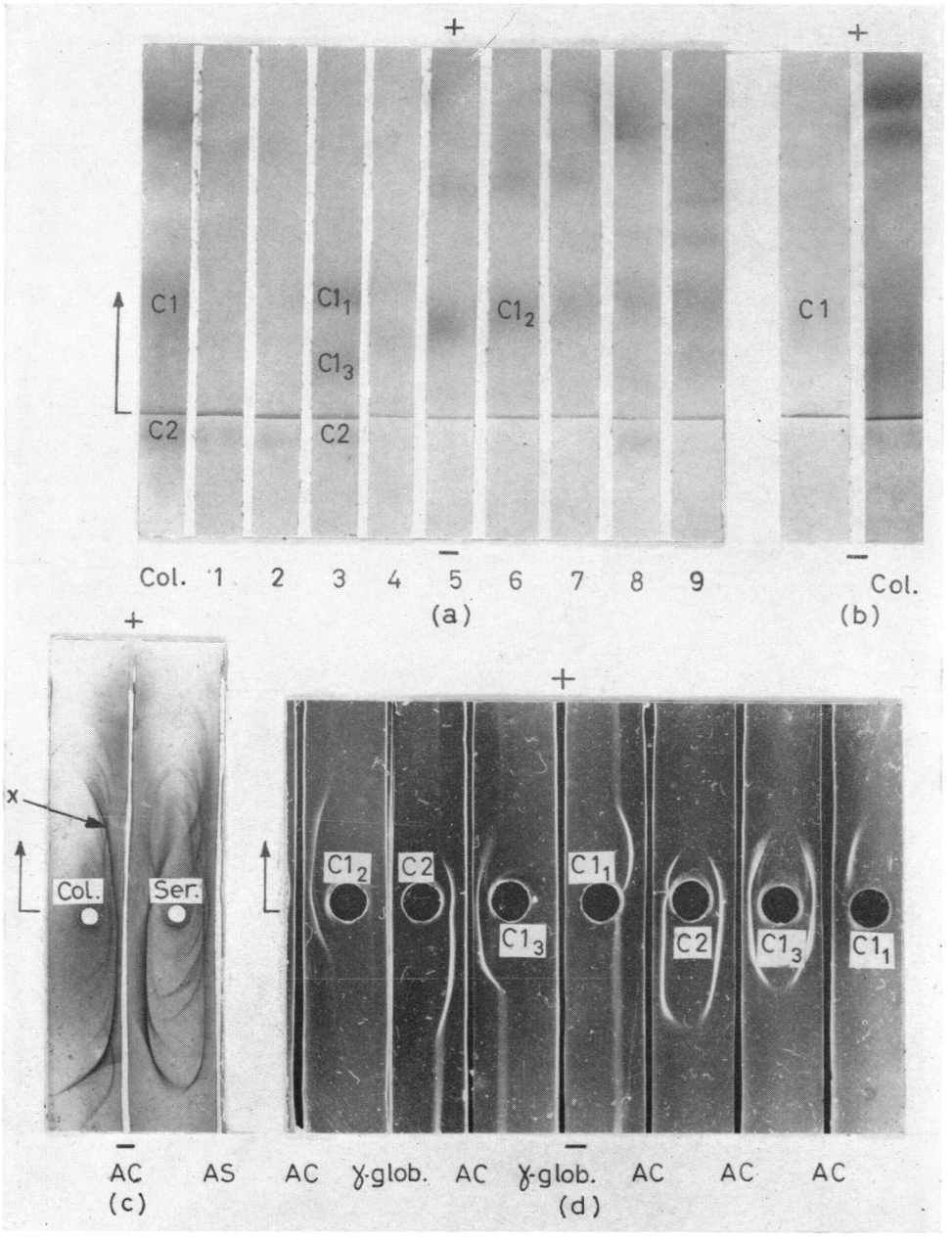

FIG. 3. Starch gel electrophoresis of (a) colostrum (Col) and fractions 1–9 collected by continuous gradient elution from a DEAE-cellulose column; (b) colostrum (Col), and a fraction (C1), prepared by DEAE-cellulose fractionation and subsequently used for antigenic analysis (see Figs. 12 and 13); (c) immunoelectrophoresis of bovine colostrum and serum against rabbit anti-bovine colostrum (AC) and rabbit anti-bovine serum (AS). The fastest colostral immune globulin is shown at 'x'; (d) immunoelectrophoresis of starch gel cut-outs from DEAE-cellulose fractions 3 and 6 shown at (a). The immunological cross-reactivity is shown between components $C1_1$, $C1_2$, $C1_3$ and C2 and Armour γ-globulin (γ-glob.).

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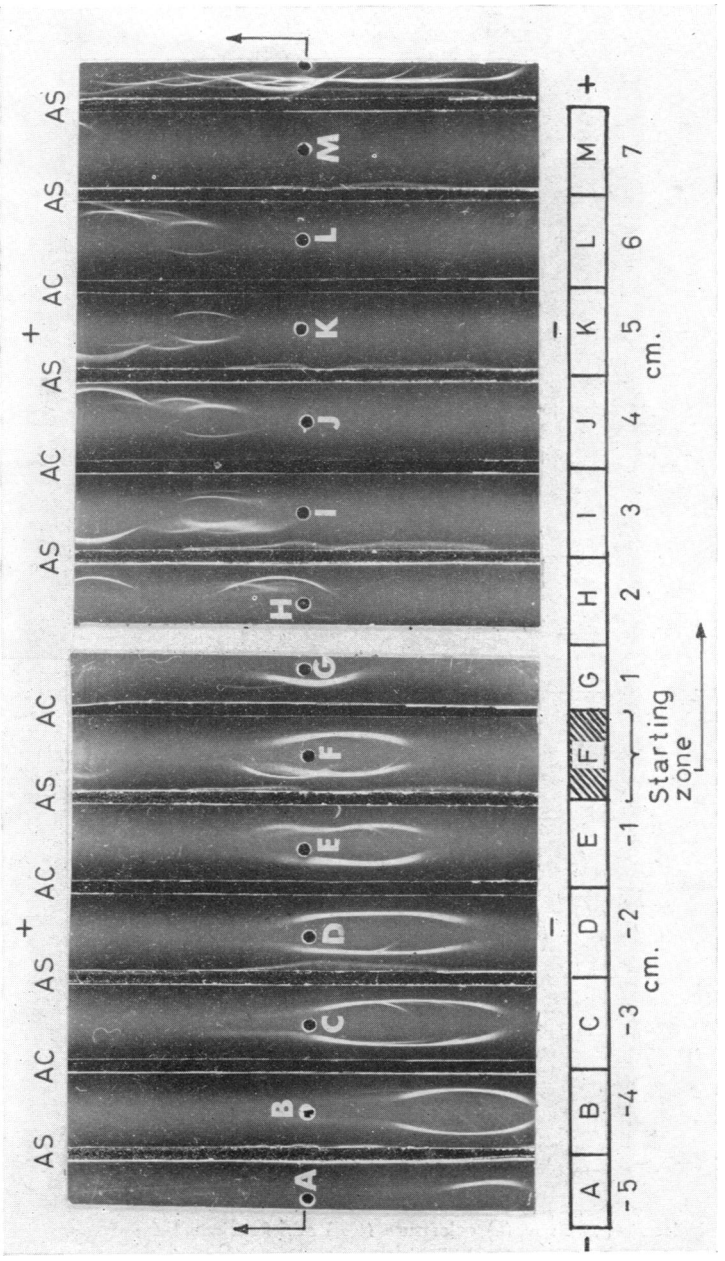

FIG. 5. A–M represent fractions examined immunoelectrophoretically after elution from segments 1 cm. wide of a starch grain block on which E4 bovine serum proteins had been electrophoretically separated. AS and AC represent rabbit anti-bovine serum and anti-colostrum respectively.

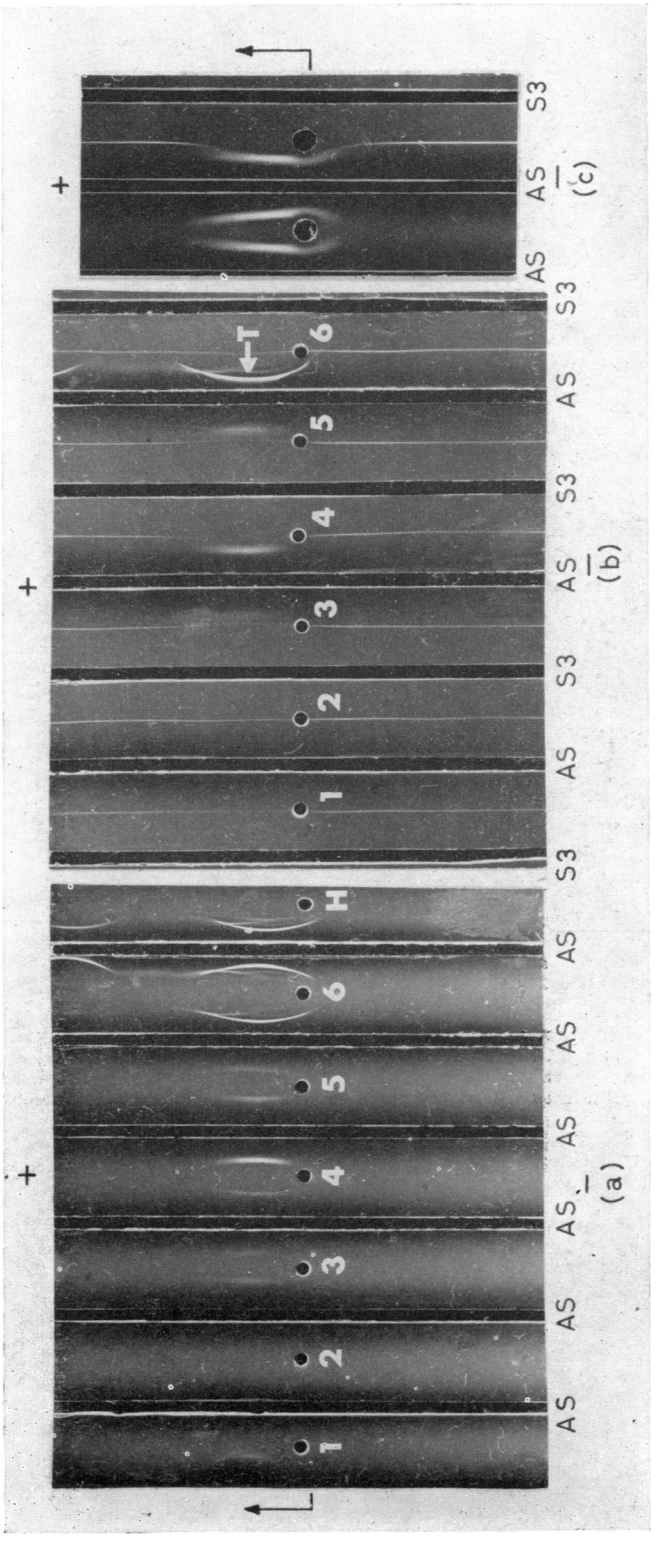

FIG. 7. (a) Immunoelectrophoresis of Sephadex G200 pools 1–6 (see Fig. 6) and starting material H (see Fig. 5) against rabbit anti-bovine serum (AS) and (b) against bovine serum immune globulin (S3), isolated electrophoretically and shown in Fig. 5 as fraction A; and rabbit anti-bovine serum (AS); (c) a fraction derived similarly to those shown at (a) and comparable to fractions 4 and 5 at (a), but at higher concentration.

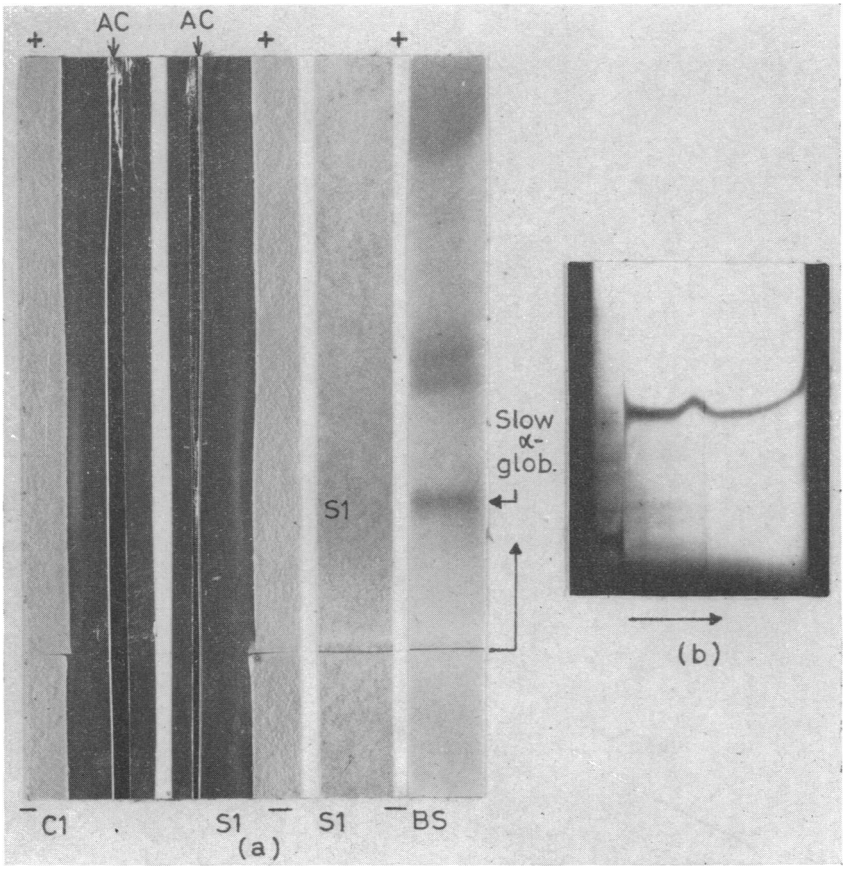

FIG. 8. (a) Starch gel electrophoresis (phosphate buffer, pH 7·6) and starch gel immunoelectrophoresis of pool 4 (S1) (see Fig. 7) and C1 isolated from colostrum (see Fig. 3(b), C1). The C1 and S1 components were developed against rabbit anti-bovine colostrum (AC). Starch gel electrophoresis of bovine serum is shown at BS; (b) ultracentrifuge pattern for pool 4 (see Fig. 7) after 56 minutes at 52,640 rev./min. t = 17·4°.

in E4 maternal serum, particularly those moving towards the anode in agar immunoelectrophoresis, compared with colostrum. A further fractionation of bovine serum was therefore undertaken to isolate and concentrate any immune globulin with an electrophoretic mobility in agar gel comparable to those present in the C1 component of colostrum.

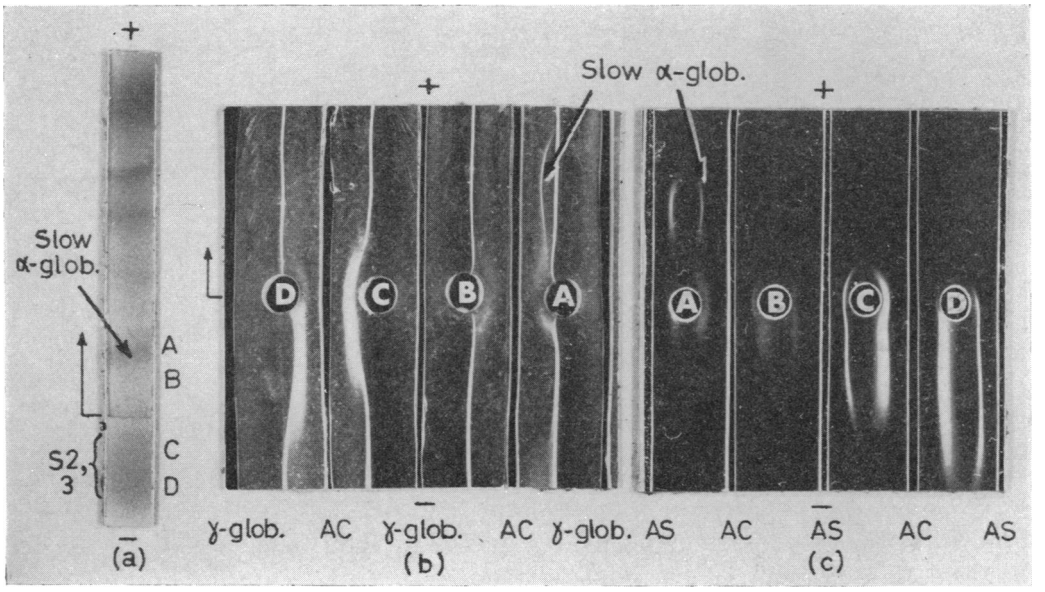

FIG. 4. Immunoelectrophoresis of cut-outs (A–D), derived from bovine serum proteins separated by starch gel electrophoresis (phosphate buffer, pH 7·6) shown at (a), against (b) rabbit anti-bovine colostrum (AC) and bovine Armour γ-globulin (γ-glob.) and (c) against rabbit anti-bovine serum (AS) and rabbit anti-bovine colostrum (AC).

Starch Grain Fractionation E4 Maternal Bovine Serum

Approximately 10 ml. of serum, derived from cow E4 during the 6 weeks prior to calving, was subjected to electrophoresis in a starch grain block. Subsequently, sections 1 cm. wide were removed, eluted and examined by immunoelectrophoresis. The results (Fig. 5) suggested that fraction 'H' could contain immune globulin with a mobility in agar gel comparable with the fastest immune globulin present in the C1 component (shown at 'x' in Fig. 3c). The fraction 'H' eluate was further fractionated on a column of Sephadex G200 (Fig. 6). The eluted fractions were pooled as indicated in Fig. 6 and after concentration, these pools were examined by immunoelectrophoresis. The pattern was developed with anti-bovine serum (Fig. 7). Pools 4 and 5 gave a single arc which extended from the well towards the anode, comparable in mobility with the 'x' portion of the immunoelectrophoretic C1 immune globulin arc (Fig. 3c), i.e. these pools represented in terms of electrophoretic mobility an S1 fraction. Fig. 7(b) shows that these pools gave a reaction of only partial antigenic identity with the slowest serum immune globulin (S3) previously isolated by starch grain electrophoresis (i.e. fraction 'A', Fig. 5).

Pool 6 (Fig. 6), from its pink colour, retardation on Sephadex gel, and immunoelectrophoretic pattern, was shown to be mainly transferrin, which serves to indicate the comparable mobility of the immune globulin in pool 4 (Fig. 7). The further examination of

the immune globulin, isolated from serum E4 and present in pool 4 (Fig. 7), by starch gel electrophoresis and by immuno-diffusion from the starch gel into agar, showed similarities to the comparable immune globulin (i.e. C1) in colostrum (Fig. 8a). Fig. 8(b) shows the analytical ultracentrifuge pattern for serum pool 4, only one component being

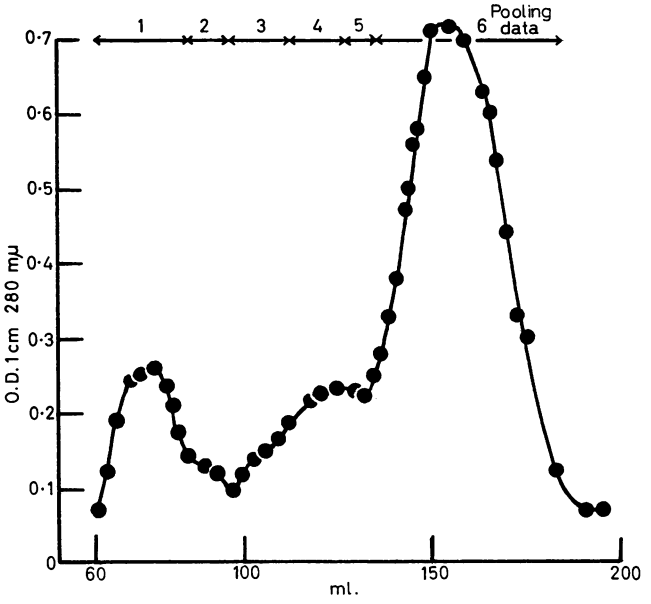

FIG. 6. Sephadex G200 elution pattern for fraction 'H' (Fig. 5) and pooling data.

detected, with a sedimentation of 7·39S. Thus, pool 4 derived from serum E4 has characteristics comparable with the fastest immune globulin present in the C1 component of bovine colostrum and may be termed S1; however, in colostrum, the protein is present at considerably higher concentration.

The low concentrations in E4 serum of the fast immune globulins could arise as the result of selective concentration by the mammary gland with depletion in the serum. Alternatively, these globulins could be synthesized by the mammary gland. Therefore, an attempt was made to isolate S1 immune globulins from a pooled sample of serum derived from steers.

DEAE-Cellulose Fractionation of Steer Serum

Starch gel electrophoresis of whole steer serum failed to detect S1 immune globulins comparable in mobility with the immune globulins in the C1 component. This was largely due to contaminating proteins since fractions from steer serum eluted from a DEAE-cellulose column (Fig. 9) contained considerably more protein with electrophoretic mobility characteristics corresponding to the C1 component of the immune lactoglobulins than did fractions similarly derived from E4 serum (Fig. 10a). Immunoelectrophoresis of the steer serum fractions (Fig. 10b) identified this protein as S1 immune globulin covering a mobility range comparable with that observed for all the immune globulins in the C1 colostral component.

Hanson (1960) suggested that β_{2A}-globulin contributes the major proportion of the

immune globulin of human colostrum and Montreuil, Chosson, Havez and Mullet (1960) showed that human β_{2A} moves towards the anode in starch gel. The conditions used for starch gel electrophoresis in the present study showed similar relationships between human and bovine serum and colostrum systems (Pierce, 1962). Therefore it seemed possible that the C1 component could include material analogous to the β_{2A}-globulin in human serum. Further experiments were carried out to examine this possibility.

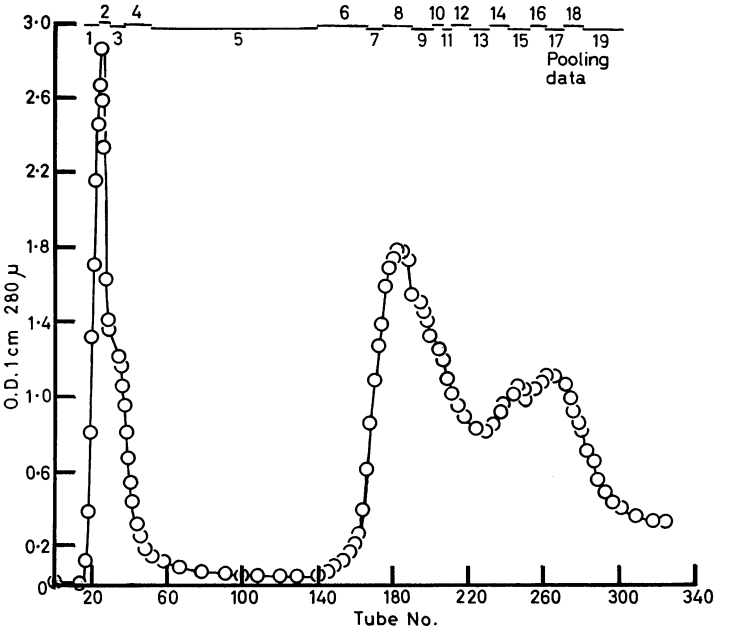

FIG. 9. DEAE-cellulose, continuous gradient, elution pattern for a pooled steer serum, and pooling data.

THE CHEMICAL BEHAVIOUR OF BOVINE IMMUNE GLOBULINS TO ZINC PRECIPITATION

Heremans (1960) showed that zinc ions selectively precipitate γ-globulin from human serum, giving a relative enrichment of β_{2A}-globulin in the supernatant. An immune β-globulin in rabbit colostrum has been similarly purified (Feinstein, 1963).

The same method was applied to bovine serum and colostrum and some of their immune globulin fractions. However, there was no apparent enrichment of any particular immune globulin component in the serum or colostrum as judged by the immunoelectrophoretic patterns of the supernatants (Fig. 11). This was borne out by the approximately equal precipitability of the purified immune globulins associated with fractions S3, S1, C2 and C1 (Fig. 12).

A FURTHER INVESTIGATION OF THE ANTIGENIC RELATIONSHIPS BETWEEN THE BOVINE IMMUNE GLOBULINS

Some of the main immune globulin components isolated and characterized from bovine colostrum and serum were selected for a more detailed examination of their antigenic relationships. The immunoelectrophoretic patterns for the particular fractions selected

are shown in Fig. 12. The C1 and C2 fractions were derived from a DEAE-cellulose fractionation of colostrum and comprised the break-through fraction (C2), similar to

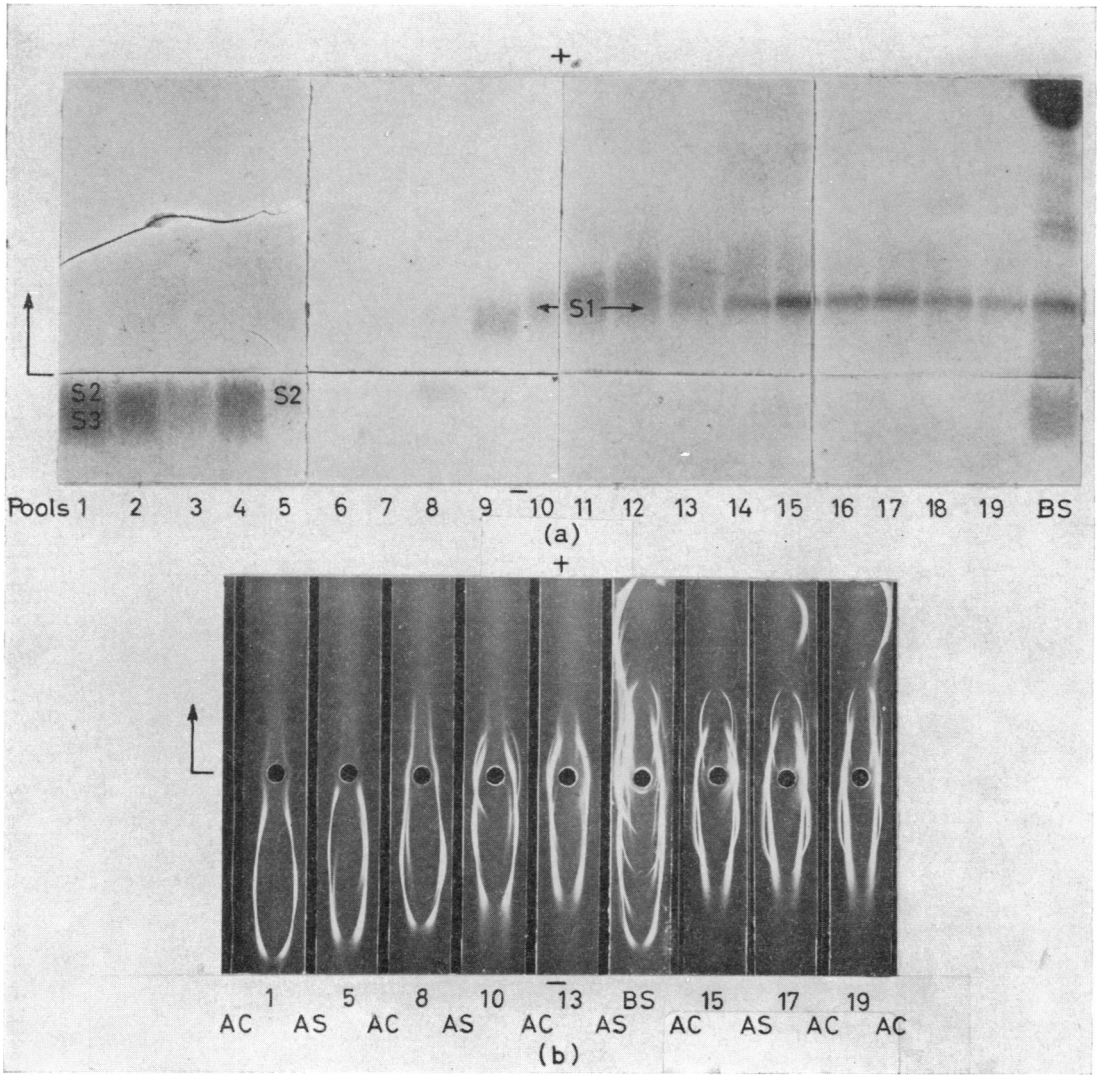

FIG. 10. (a) Starch gel electrophoresis and (b) immunoelectrophoresis for DEAE-cellulose fractionation pools 1–19 (see Fig. 9); BS, bovine serum; AS, rabbit anti-bovine serum; AC, rabbit anti-bovine colostrum.

pool 1, Fig. 3, and a fraction from the first peak eluted after the start of the gradient. The latter fraction, C1, was selected after reference to its starch gel (Fig. 3b fraction C1) and immunoelectrophoretic pattern (Fig. 12).

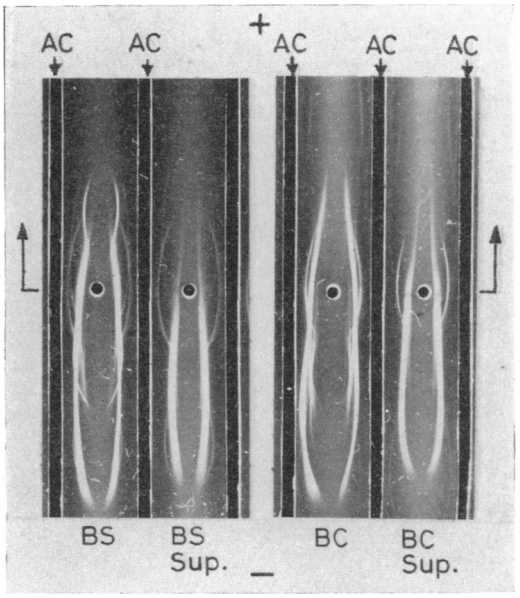

FIG. 11. Immunoelectrophoresis of bovine serum (BS) colostrum (BC) and their respective supernatants (BS sup. and BC sup.) after zinc precipitation. AC, rabbit anti-bovine colostrum.

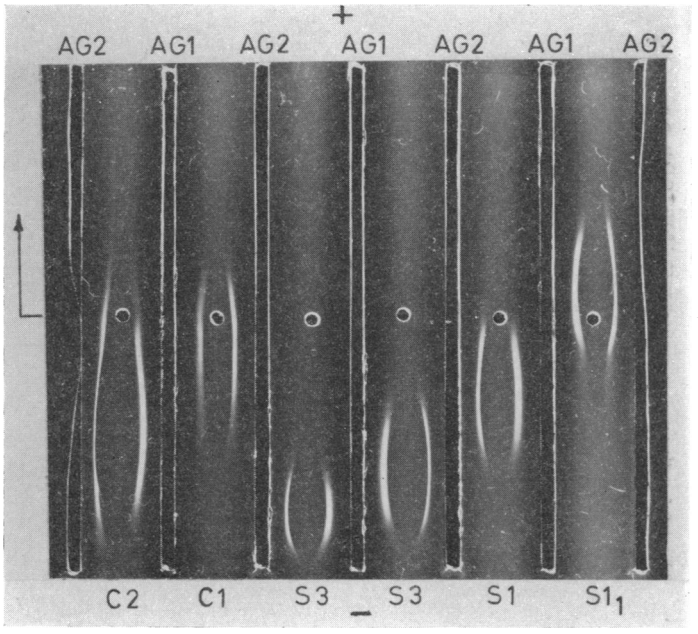

FIG. 12. Immunoelectrophoresis showing the relative mobilities for fractions isolated from bovine serum (S1, S1$_1$, S3) and colostrum (C1, C2) and developed with two rabbit anti-bovine serum immune globulin sera with overlapping but different specificities, AG1 and AG2. C2 = Similar Fig. 3, pool 1; C1 = Fig. 3(b), fraction C1; S3 (left) = Fig. 5, fraction A; S3 (right) = Fig. 10, fraction 1; S1 = Fig. 10, fraction 10; S1$_1$ = Fig. 7, fraction 4.

The two rabbit anti-bovine immune globulin sera used were of different, but overlapping, specificities. This is shown in Fig. 13(i) where they were used to develop the immunoelectrophoretic patterns of bovine colostrum. The patterns show that antiserum AG1 is deficient with respect to AG2 in antibody to faster, e.g. C1-like, immune globulins, whilst antiserum AG2 is deficient in antibody to the slower immune globulin. These two

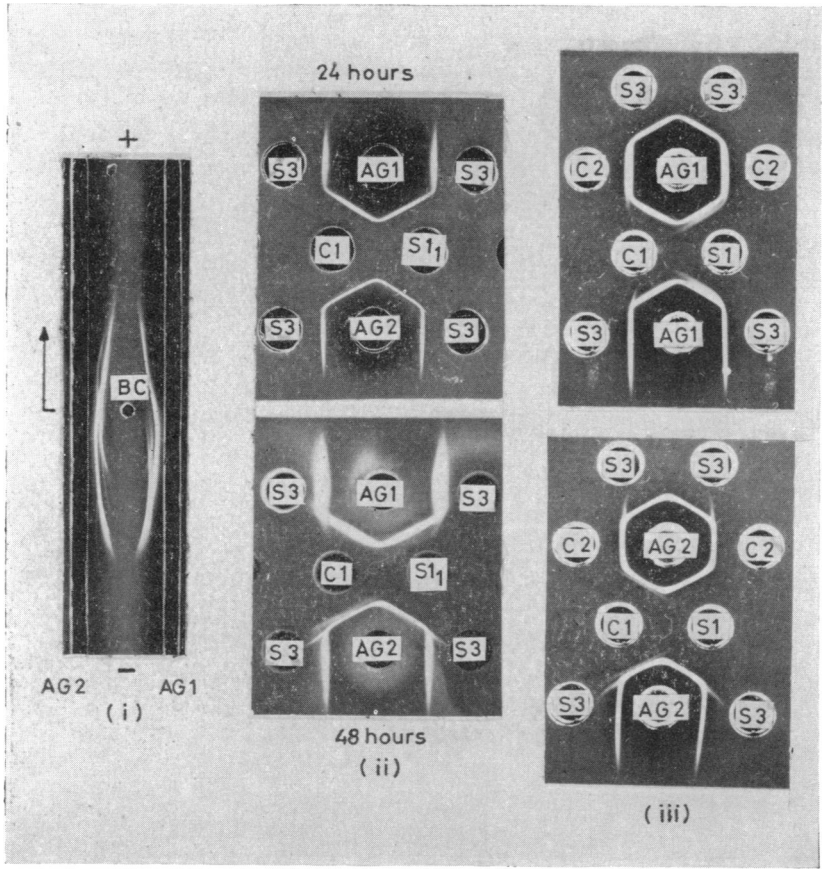

FIG. 13. (i) Immunoelectrophoresis of bovine colostrum (BC) showing the overlapping specificities for two rabbit anti-bovine serum immune globulin sera (AG1, AG2). (ii) and (iii) double diffusion in agar of fractions isolated from bovine serum (S1, $S1_1$, S3) and colostrum (C1, C2) developed with rabbit sera AG1 and AG2. In (ii) S3 = Fig. 5, fraction A; $S1_1$ = Fig. 7, fraction 4; C1 = Fig. 3(b), fraction C1. In (iii) S3 = Fig. 5, fraction 4; S1 = Fig. 10, fraction 10; C2 = similar Fig. 3, fraction 1, C1 = Fig. 3(b), fraction C3. All these fractions in (ii) and (iii) are examined immunoelectrophoretically in Fig. 12.

antisera AG1, AG2 were also used to examine, by double diffusion, the antigenic relationships of the various selected immune globulin components. Fig. 13(ii) shows that, with either antiserum, reactions of identity were obtained between the fastest S1 ($S1_1$) (see Fig. 12) and the C1 (see Fig. 12) components. However, spurring was observed between the precipitin lines of the slowest serum immune globulins (S3) (see Fig. 12) and each of the fast components (C1, S1). When antiserum AG1 was replaced by AG2 the directions

of spurring were reversed in a manner consistent with the spurrings observed in Fig. 13(i). The fastest S1 component ($S1_1$) is thus apparently antigenically identical to the main C1 component.

When the major S1 component was compared with the C1 component, again reactions of identity were observed with either antiserum (Fig. 13(iii)). Thus, no antigenic difference is observed between S1 components anodic ($S1_1$) or cathodic in agar. With antiserum AG2 the slowest serum immune globulin (S3) component appears, from the spurs which develop, to be antigenically deficient in relation to the S1 or C1 components, or to C2 component (Fig. 13(iii)). Antiserum AG2 also gives reactions of identity with C2 and with S1 or C1 (Fig. 13(iii)).

In contrast, however, antiserum AG1 shows a reaction of identity between S3 and C2 components (Fig. 13(iii)); the S3 or C2 components no longer appear identical to S1 or C1 components, and their line appears to fuse with two lines in the patterns of both S1 and C2 components. Since the S3 component isolated from starch block electrophoresis, whether examined by immunoelectrophoresis or simple diffusion, has never given other than a single line using many antisera, it seems possible that the antigenic determinants on S3 component are distributed in the faster immune globulins between two different molecular species. This is in line with the immunoelectrophoretic pattern of bovine colostrum developed with antiserum AG1 (Fig. 13(i)); the forward spur may indicate the fusion of two arcs in the slowest immune globulin (S3) region.

Gamma-globulin preparations are known to be families of structurally related molecules. The formation of a single precipitin band, between a γ-globulin preparation and an antiserum, must be dependent on a continuous sequence of overlapping antigens present in the preparation, and on the presence of a corresponding range of specificities in the antiserum. Should any 'linking' specificities be absent from either antigen preparation or antiserum, multiple precipitating systems could result. This may explain why heterologous antisera are frequently observed to form multiple lines with γ-globulin preparations which give a single line with the homologous antiserum. It may also explain the above observations with bovine γ-globulin.

On the basis of the relationships which have been described, it may be seen that at least three types of immune globulin can be distinguished serologically among the components isolated from serum and colostrum. The S1 ($S1_1$ and S1) and C1 components are antigenically identical.

PERMEABILITY OF THE INTESTINE OF THE NEWLY BORN CALF TO THE IMMUNE GLOBULINS

Evidence has been presented which shows that the mammary gland preferentially concentrates certain of the immune globulins and that these can be identified by their electrophoretic behaviour. A further investigation was carried out to determine whether the intestinal mucosa of the new-born calf showed any comparable selectivity.

A newly born calf was fed colostrum containing virtually no protein comparable with the S3 component (see Fig. 1) and containing the C1 and C2 components in the ratio of 1 : 1·8 respectively. If the slow α-globulin in the post-colostral calf serum, which overlaps the passively acquired C1 component, was allowed for, then the ratio of the C2 to C1 component, passively acquired by the calf, was 1:2·7; a difference which cannot be considered as significant. Since the mammary gland discriminated between the S2 and S3 components, in a further experiment 2 litres of normal adult bovine serum were fed to a

calf during the first 14½ hours of life. The distribution of protein between components S2 and S3, in the serum fed and the serum of the newly born calf after feeding, was similar (Fig. 14). This was confirmed from the scanned patterns of the immune globulins fed and of the serum of the calf after ingesting these proteins.

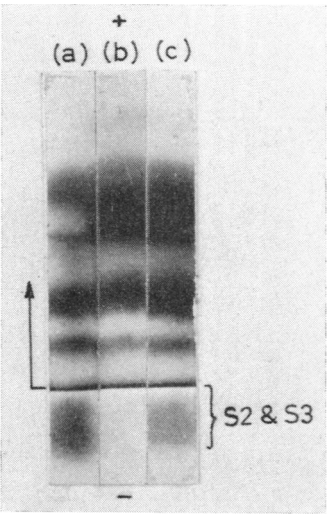

FIG. 14. Starch gel electrophoresis: (a) adult bovine serum, fed to calf in 4 × 500 ml. amounts during first 14½ hours of life; (b) calf serum pre-feeding (5 minutes after birth); (c) calf serum post-feeding (17½ hours after birth)

DISCUSSION

Recent evidence indicates that the immune lactoglobulin is derived unaltered from the circulating plasma proteins. Askonas *et al.* (1954), using isotopically labelled amino acids in the rabbit and goat, and Larson and Gillespie (1957) using ^{14}C (as Na_2CO_3) in cattle, showed the transference of serum immune globulin to the colostrum without degradation and resynthesis. These findings were supported by data derived from histological methods (Dixon *et al.*, 1961; Feldman, 1961). Blakemore and Garner (1956) and Garner and Crawley (1958) showed that homologous γ-globulin, given intravenously to pregnant cattle, was transferred to the mammary secretions during the weeks immediately prior to calving. The antibody level was 13 times higher in the colostrum than in the maternal serum. A simple splitting of the molecule with the retention of antibody activity is unlikely as the sedimentation behaviour of the immune lactoglobulin is similar to bovine γ-globulin (Johnson and Pierce, 1959). It seems probable that differences in the amino acid composition and in ultra-violet absorption spectra between serum immune globulin and immune lactoglobulin (Smith and Coy, 1946; Smith, Green and Bartner, 1946), are related to differences, shown by the present results using more refined fractionation and electrophoretic techniques, between the make-up of the immune lactoglobulin and serum globulin components.

The present findings show that immune globulins of similar electrophoretic behaviour and antigenic relationships are present in bovine serum and colostrum. Assuming a plasma protein origin for the colostral immune globulins, the mammary gland can be shown not only to discriminate between the major serum proteins but also between electrophoretically distinguishable immune globulin components. Thus, there is an almost complete exclusion from the immune lactoglobulins of the slowest electrophoretic component of the serum immune globulin (S3). No similar selective absorption was shown by the intestinal mucosa between the S2 and S3 components present in serum when this was fed to a newly born calf in place of colostrum; both components were absorbed with equal facility. This is in keeping with other observations showing the absence of any well-defined selectivity by the intestine of the newly born calf (Bangham, Ingram, Roy, Shillam and Terry, 1958; Pierce, 1959, 1961; Pierce, Risdall and Shaw, 1964).

About 50 per cent of the total colostral protein showed a mobility in starch gel comparable with the C1 component although a component with the same microheterogeneity could not readily be shown in bovine serum. However, traces of such a protein would be obscured in the starch gel pattern for serum by the slow α- and β-globulin components. A trace of precipitate, extending towards the anode from the main immune globulin arc, was observed on the immunoelectrophoretic examination of bovine serum. This protein (S1) was isolated from serum and shown to have a partial immunological cross-reactivity with electrophoretically slow migrating serum immune globulin (S3) and to be antigenically identical with the fast colostral component (C1). This protein also showed a sedimentation behaviour characteristic of serum γ-globulin (~7S). Therefore, the C1 protein could be derived directly from the plasma and would then indicate a marked selective concentration in the colostrum of the S1 plasma immune globulin component.

These findings suggest an increasing selection by the mammary gland for plasma immune globulins with increasing electrophoretic mobility. Thus, although the intestine of the newly born calf shows no selectivity in absorptive capacity, a high degree of selectivity has previously taken place in the mammary gland during the accumulation of the immune lactoglobulins that are subsequently made available in colostrum to the suckling calf.

If bovine immune globulin had only a microheterogeneity and not the electrophoretic heterogeneity shown, such a selectivity between faster and slower molecular species would be difficult to explain. However, the differences shown in the relative concentrations of the fractions of immune globulin and immune lactoglobulin are related to groups of proteins showing discontinuous electrophoretic mobilities. Thus, major structural differences could occur between different components and these could play a significant part in determining the differences in transmission within the mammary gland. There are several examples which suggest that the transmission of macromolecules with antibody activity is related to their location within the immune globulin components. This was strongly indicated by Halliday and Kekwick (1961), who showed that the gut of the young rat appeared to distinguish selectively between antibodies in sera produced from a single species immunized with *Salmonella pullorum* antigen; the only differences in the serum fed were in the duration of the immunization at the time of bleeding and the serum antibody titre. Fractionation experiments showed a shift, with immunization, in the location of antibody activity within the serum immune globulins. A change of this nature is well known, for example diphtheria antitoxin in equine serum translates from the γ_2 to the γ_1 during the course of immunization (Kekwick and Record, 1941; Cinader and Weitz, 1953).

Changes such as these may also account for the preferential transmission from rat plasma to milk of antibodies stimulated by primary immunization compared with those by secondary immunization (Biswas, 1961). Similarly, certain serum antibodies are preferentially transmitted across the human placenta, antibodies to poliomyelitis pass the placental barrier readily (Perkins, Yetts and Gaisford, 1958), whereas those to *Escherichia coli* pass very poorly (Sussman, 1961); typhoid 'H' agglutinins are more readily transmitted than the 'O' agglutinins, although 'O' agglutinins are preferentially concentrated in the colostrum (Timmerman, 1931). Brambell, Halliday and Hemmings (1961) showed that ^{131}I-labelled bovine γ-globulin was absorbed into the circulation of the newly born rat in substantial amounts although the bacterial agglutinins were excluded. The bovine serum immune globulin fractions S1–S3 isolated from bovine antiserum to ferritin do not appear to have the same immune precipitating activity (these results will be reported elsewhere).

Thus, a selective transmission across any membrane in a manner comparable with the bovine mammary gland, could fractionate the immune globulin components and restrict or permit the passage of antibodies according to their location within the immune globulins.

Further experiments are being carried out to relate the separated polypeptide chains of the different bovine immune globulins, and to locate 'recognition units' which may be significant in selective transport, similar to those already described by Brambell *et al.* (1960).

ACKNOWLEDGMENTS

We should like to thank Dr. P. Lachmann of the Department of Pathology, University of Cambridge, for kindly giving us the antisera AG1 and AG2 and to thank Mr. D. Hardman and Mr. J. Herbert for technical assistance.

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