*Immunology*, 1972, **23**, 299.

# Intestinal Secretion of Immunoglobulins in the Preruminant Calf

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(*Received* 30*th December* 1971)

**Summary.** Intestinal immunoglobulins have been examined in secretions obtained from Thiry-Vella loops prepared in the mid-jejunum of six calves. Free secretory component and 11S IgA were demonstrated. Immunofluorescent studies of intestinal tissue confirmed the presence of a secretory IgA system similar to that described in other species. However quantitative assays showed that IgA was not at any time the predominant immunoglobulin in intestinal secretions. IgM consistently exceeded the level of IgA by approximately two-fold and its localization in lymphoid cells of the lamina propria and crypt epithelial cells suggested that IgM may normally play an important role in local intestinal defence in the calf.

IgG1 appeared in consistently low levels in intestinal secretions in spite of being the major serum immunoglobulin.

## INTRODUCTION

The best defined characteristic of bovine secretory immune systems has been the selective transfer of IgG1 to colostrum and milk (Larson, 1958; Murphy, Aalund, Osebold and Carrol, 1964; Pierce and Feinstein, 1965). This immunoglobulin has received considerable attention in bovine immunology and until recently there has been little or no evidence for a local secretory immune system mediated by IgA as in other species (Sullivan, Prendergast, Silverstein and Tomasi, 1969). Thus the recent identification of IgA and secretory component in the bovine (Mach, Pahud and Isliker, 1969; Porter and Noakes, 1970; Butler, 1971) and the demonstration of serological cross-reaction of the immunoglobulin with human IgA (Vaerman, 1970) has stimulated considerable interest in its role in local immune defence mechanisms in this species.

Quantitative studies have shown that in certain secretions in the bovine, IgA may be the predominant immunoglobulin present (Mach and Pahud, 1971). Furthermore, in studies of a calf prepared with a Thiry-Vella loop of the small intestine we have found that the the level of IgA in intestinal secretion was similar to that previously described in the pig, (Porter and Noakes, 1970). However Vaerman (1970) failed to detect many IgA type cells in the lamina propria of bovine intestinal tissues. Thus the question of bovine intestinal immunoglobulin secretion presents an interesting problem in relation to what is known for monogastric species.

In this paper, the results of detailed investigations of the nature and amount of immunoglobulins secreted in the small intestine of the preruminant fistulated calf are presented together with immunohistochemical studies of the intestinal mucosa with particular reference to the localization of immunoglobulins and the secretory component. The

studies have been conducted in an attempt to explore the possible role of specific immunoglobulins in early intestinal defence in the bovine.

## MATERIALS AND METHODS

*Isolation of immunoglobulins*

The isolation of bovine immunoglobulins and the preparation of $\gamma_1$-, $\gamma_2$-, $\alpha$- and $\mu$-chain specific antisera have been described previously (Porter and Noakes, 1970).

*Isolation of secretory component*

Free secretory component was isolated from bovine milk on the basis of previous observations (Mach, 1970; Porter and Noakes, 1970; Porter, 1971) that when milk whey was subjected to ion exchange chromatography on DEAE cellulose secretory component appeared in the fall through fraction eluting with 0·01 M phosphate buffer pH 7·6. Secretory component was precipitated from this fraction with 2·05 M $(NH_4)_2SO_4$ redissolved in 0·1 M Tris-HCl buffer pH 7·2 and further purified by recycling chromatography on Sephadex G-150 (90 × 2·5 cm).

*Microelectrophoresis*

Protein fractions were examined by immunoelectrophoresis using antisera raised in New Zealand White rabbits. Disc electrophoresis in polyacrylamide gels was done by the method of Orstein and Davies (1964).

*Quantitative estimation of immunoglobulins*

Immunoglobulins were assayed by the radial immunodiffusion technique of Mancini, Cabonara and Heremans (1965).

*Chromatographic methods*

Gel filtration chromatography was carried out on Sephadex G-200 columns (45 × 2·5 cm and 90 × 2·5 cm) using 0·85 per cent NaCl in 0·1 M Tri-HCl buffer pH 7·2.

Anion exchange chromatography was carried out on diethyl-aminoethyl cellulose as part of the procedure for purification of immunoglobulins (Porter and Noakes, 1970).

*Ultracentrifugation*

Density gradient ultracentrifugation was carried out by the isokinetic sucrose gradient technique of Noll (1967) using an MSE 50 superspeed centrifuge. Protein samples (0·2 ml) were layered onto the surface of 2·8-ml volumes of a 10–30 per cent sucrose density gradient. The samples were centrifuged at 200,000 ***g*** for 19 hours.

A Beckman model E centrifuge equipped with phase plate Schlieren diaphragm was used in ultracentrifugal analysis of fractions. Sedimentation coefficients were determined at 20° at a speed of 59,780 rev/min.

*Immunofluorescent histochemistry*

The technique of preparing fluorescein isothyocyanate (FITC) conjugated rabbit antisera and detection of immunoglobulins in cryostat sections of intestinal tissue has been described (Allen and Porter, 1970).

Cell counts were performed on paraffin sections prepared by the method of Saint Marie

(1962) and stained with fluorescein conjugated rabbit anti-globulins with specificity for $\alpha$ or $\mu$ chains and secretory component. More precise counts could be obtained from specimens prepared in this way than from cryostat sections.

All fluorescent cells present in twenty fields selected at random from the lamina propria were counted using a $\times 40$ objective, 0·65 numerical aperture. Secretory component was also visualized by the indirect 'sandwich' technique (Nairn, 1969) using specific rabbit antiserum to bovine secretory component followed by sheep anti-rabbit fluorescein conjugate. Localization of immunoglobulins IgA, IgG and IgM and secretory component was studied in tissues taken from three levels of the small intestine (duodenum, jejunum, ileum) as well as from the Thiry-Vella loops.

*Experimental animals*

Thiry-Vella loops were prepared in the mid position of the jejunum of three Ayrshire calves aged 2–4 days and double re-entrant fistulae in the same region of the small intestine of three Ayrshire calves 2–5 days of age. Anaesthesia was induced by the intravenous injection of methohexitone sodium ('Baretal' Elanco Ltd, London) and after endotracheal intubation, was maintained with cyclopropane/oxygen.

Thiry-Vella loops 30–40 cm in length were prepared by the method of Markowitz (1954) using Perspex gutter cannulae (1·2-cm i.d., 1·5-cm o.d.). The continuity of the small intestine was restored by side to side anastomosis and the cannulae were exteriorized through stab incisions in the right flank of the calf.

Double re-entrant fistulae were prepared by a method described by Harrison and Hill (1964). The distance between the two re-entrant fistulae was 30–40 cm.

*Collection of intestinal secretion*

The secretion from Thiry-Vella loops was collected in small polythene bags attached to the end of each cannula over a period of several hours.

Secretions from calves with double re-entrant fistulae were collected from the length of intestine between the two pairs of cannulae. This was done by by-passing the short piece of intestine between the fistulae with a length of polythene tube attached to the appropriate cannulae. Thus a flow of digesta was maintained along the intestine but a short segment of intestine was open to the external bodywall and became temporarily equivalent to a Thiry-Vella loop. This loop was irrigated with physiological saline to wash away digesta contained within the lumen. Secretions were collected as described for the Thiry-Vella loop and following a period of collection the cannulae were reconnected so that a flow of digesta was maintained.

The animals were studied during the 2nd–6th week of life and fed only cows' milk so that the rumen development and function was delayed.

## RESULTS

### PROTEINS AND IMMUNOGLOBULINS IN INTESTINAL SECRETIONS

The nature and consistency of the protein profiles in secretions obtained from loops of small intestine in six fistulated calves was examined by electrophoresis in polyacrylamide gels. Fig. 1 shows that although the concentration of protein varied in different calves there was, in general, a similarity in the electrophoretic profile. The main component in the secretions was albumin, but its proportion was sufficiently low and the electrophoretic

profiles were obviously different from serum suggesting that the proteins present were the result of true secretion and not serious leakage due to inflammation or surgical trauma. The secretions of four of the six calves were clearly deficient in IgG.

The variation in protein secretion was examined over a period of more than 4 weeks (generally the length of any one animal experiment). A typical example for calf 19 is shown in Fig. 1. In the first 2 weeks protein secretion is seen to develop and after the 15th

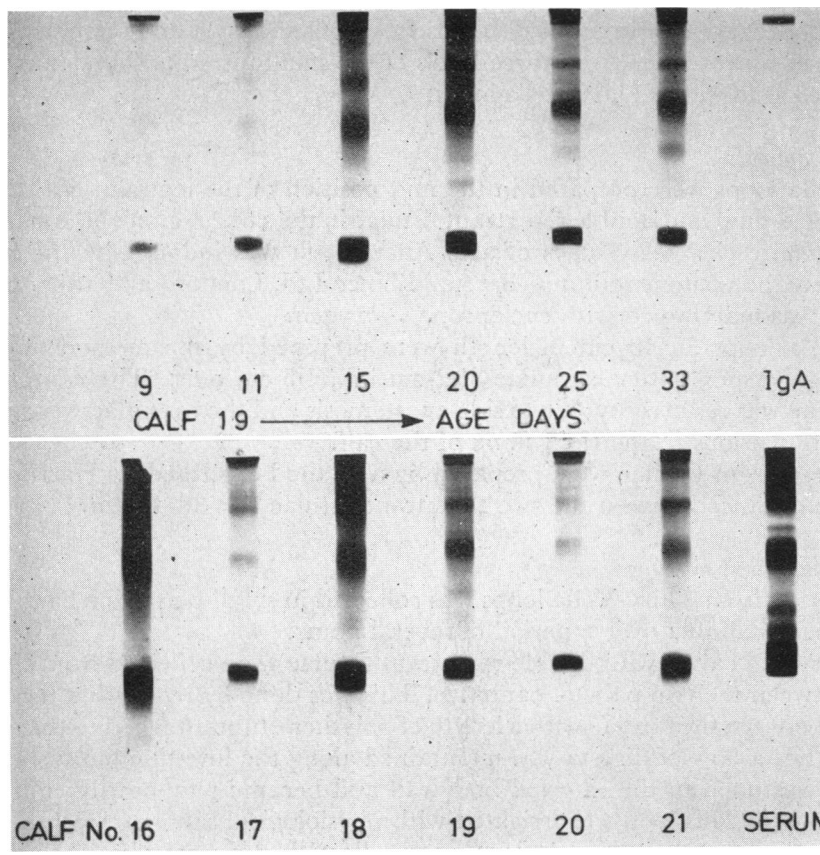


FIG. 1. Polyacrylamide disc electrophoresis of intestinal secretions obtained from fistulated calves. Upper: Change in electrophoretic protein profile in intestinal secretions from calf 19 at ages 9–33 days. Lower: Electrophoretograms of intestinal secretions from six fistulated calves compared with serum.

day a fairly consistent protein profile was obtained in which a component with the same electrophoretic characteristic as secretory IgA could clearly be defined.

The presence of serum components in intestinal secretions was examined by immunoelectrophoresis using rabbit anti-bovine serum. Immunoelectrophoretograms of intestinal secretions from four calves are compared with a calf serum pattern in Fig. 2. Approximately twelve serum components including the immunoglobulins were detectable. IgG, IgM and IgA were demonstrated by immunoelectrophoresis using specific antisera and in immunoelectrophoretograms using rabbit anti-bovine secretory IgA (Fig. 3) two precipitin lines were evident suggesting the presence of free secretory component. The reagent produced only a single line in the immunoelectrophoretogram of calf serum.

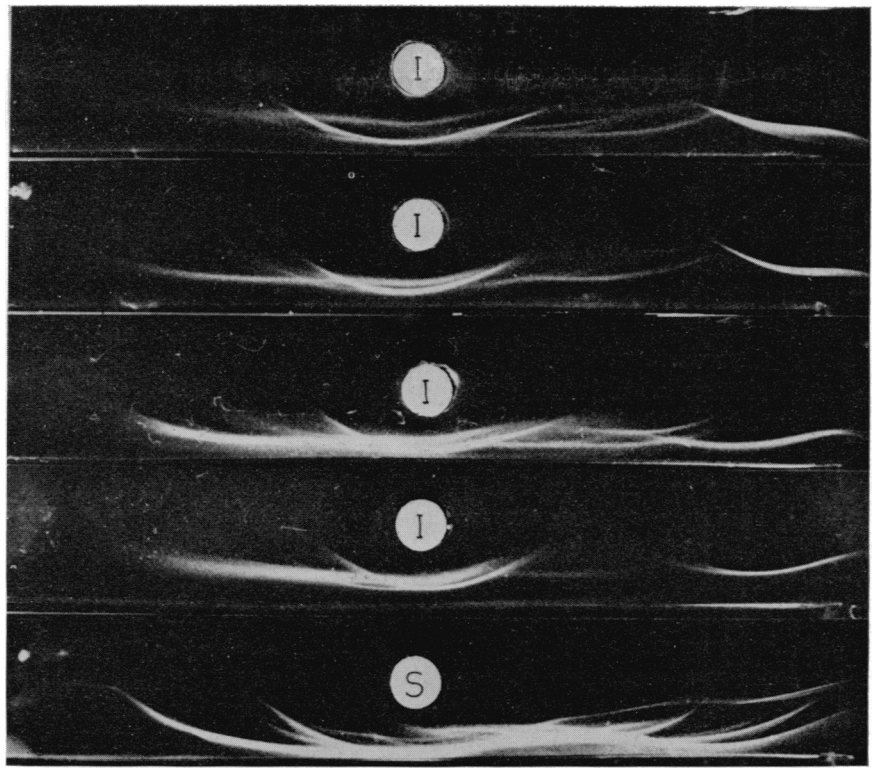


FIG. 2. Immunoelectrophoresis of intestinal secretions (I) from four calves compared with serum (S).

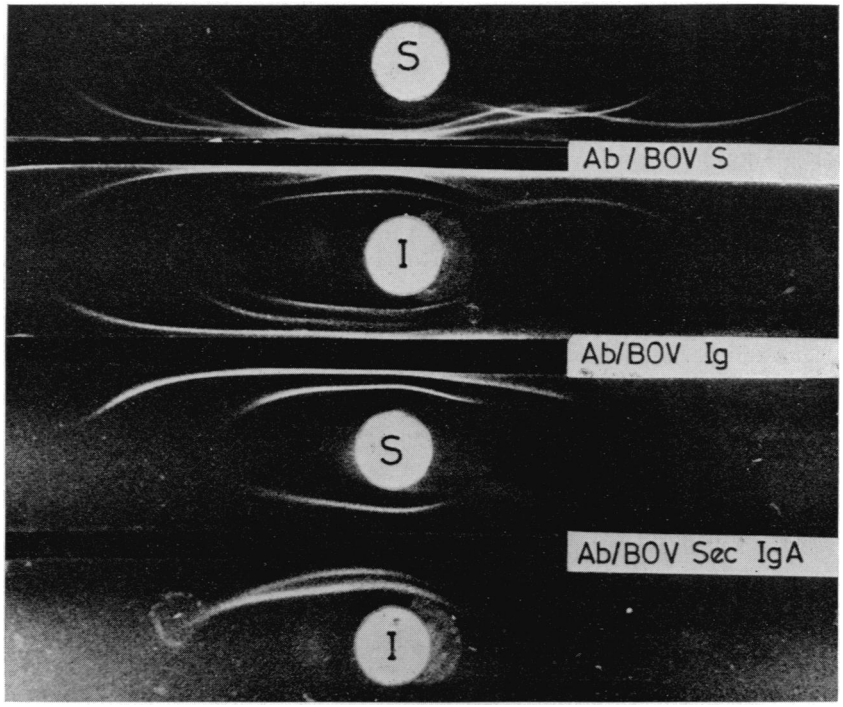


FIG. 3. Immunoelectrophoresis of calf serum (S) and intestinal secretions (I) developed with antisera of various specificity.

Gel filtration of calf intestinal secretions on Sephadex G-200 confirmed the presence of free secretory component in that the two precipitin reactions with rabbit anti-bovine secretory IgA were differentiated into high and low molecular weight fractions (Fig. 4). In immunological double diffusion studies the low molecular weight component from intestinal secretions gave a reaction of complete identity with secretory component isolated from bovine milk (Fig. 5). Similarly intestinal IgA gave a line of complete identity with bovine milk secretory IgA which also gave the classic spurring reaction with serum IgA indicative of the additional determinant secretory component, on the secreted immunoglobulin. Thus the presence of secretory IgA and free secretory component in calf intestinal secretions was confirmed.

The molecular size characteristics of the immunoglobulins shown by gel filtration (Fig. 4) were in general indicative of the lack of degradation due to any possible proteolytic activity. IgA appeared in the exclusion peaks of intestinal secretions from all the calves studied and there was no evidence of lower molecular weight forms. IgM also appeared entirely in the exclusion peak with no evidence of the 7S subunit described in previous similar studies of the pig (Porter, Noakes and Allen, 1970). The elution characteristics of IgG were such as to suggest the presence of polymeric forms in high molecular weight fractions. The molecular size characteristics of the immunoglobulins in intestinal secretions were confirmed by sucrose density gradient ultracentrifugation (Fig. 6). Intestinal IgM and IgA had the same sedimentation characteristics as the 18S and 11S standards respectively. Intestinal IgG was widespread in the centrifugation pattern appearing in fractions including 18S and 11S components as well as the normal 7S. Free secretory component appeared in fractions mainly intermediate to 4 and 7S.

Quantitative assays of immunoglobulins by radial immunodiffusion using specific $\gamma_1$-, $\gamma_2$-, $\alpha$- and $\mu$-chain antisera are given in Table 1. Contrary to general findings for secretions in other species IgA was not the predominant immunoglobulin in intestinal secretions of any of the calves and in general its level was exceeded by IgM and in two of the animals by IgG2. IgG1 appeared in consistently low levels in all the calves despite being the major immunoglobulin in the blood serum throughout the period of study. In three of the calves IgG2 was a minor component appearing in similar levels to IgG1; however in two of the animals IgG2 was the predominant immunoglobulin in the secretions considerably exceeding the level of IgM.

In most of the secretions a mucous gel formed after refrigeration. In a few samples this was separated, washed in ice cold saline and dissociated in 7 M urea containing 0·1 per cent 2-mercaptoethanol at 4° for 24 hours. Immunoglobulins were assayed in the resultant solutions after dialysis. In all cases IgA was the predominant immunoglobulin released from the mucous clot together with IgG2; IgM was either not detectable or present only in small amount.

### IMMUNOFLUORESCENT LOCALIZATION OF IMMUNOGLOBULINS IN INTESTINAL TISSUE

In tissue stained with fluorescein conjugated rabbit anti-bovine-IgA-globulin, IgA was demonstrated in the apical cytoplasm of epithelial cells in the lower region of the crypts. This reaction was observed at all levels of the small intestine, although generally it was less strong in the duodenum, than in the jejunum or ileum. There was no evidence of IgA in the epithelial cells lining the villus although the mucin covering the apical surfaces contained IgA, which provided a thin coat of bound immunoglobulin. In the

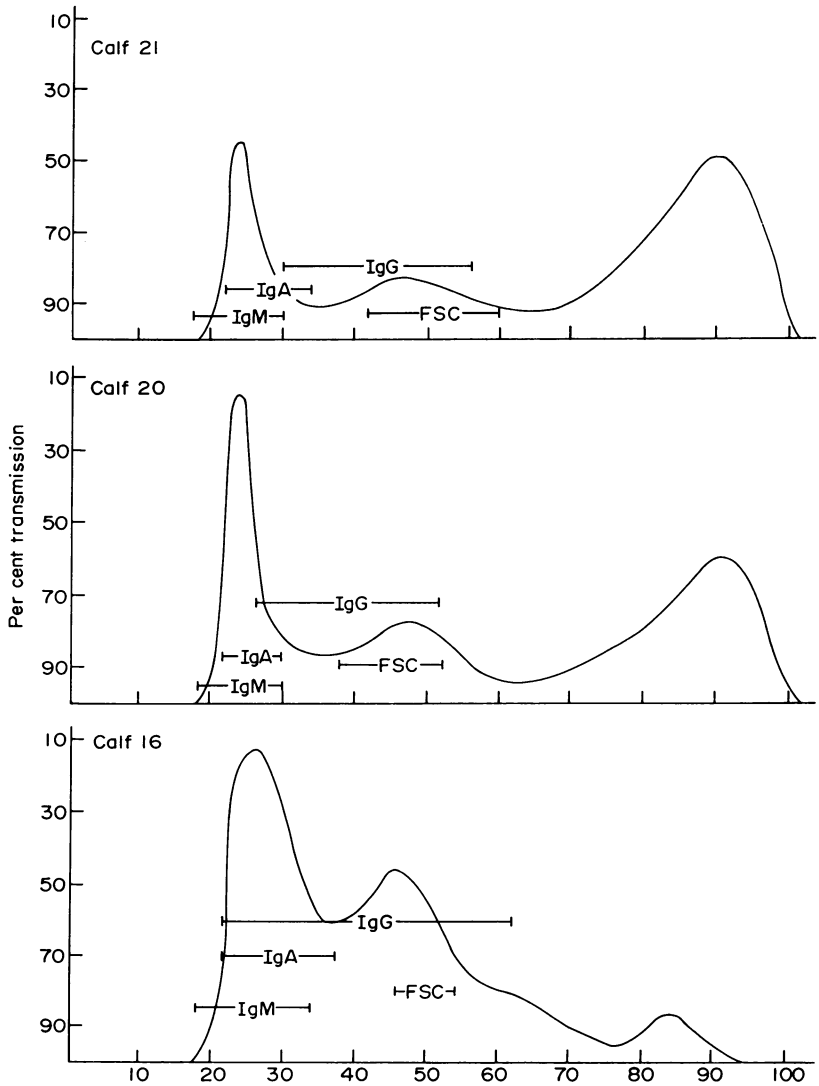


FIG. 4. Gel filtration molecular size characteristics of immunoglobulins in intestinal secretions from three calves.

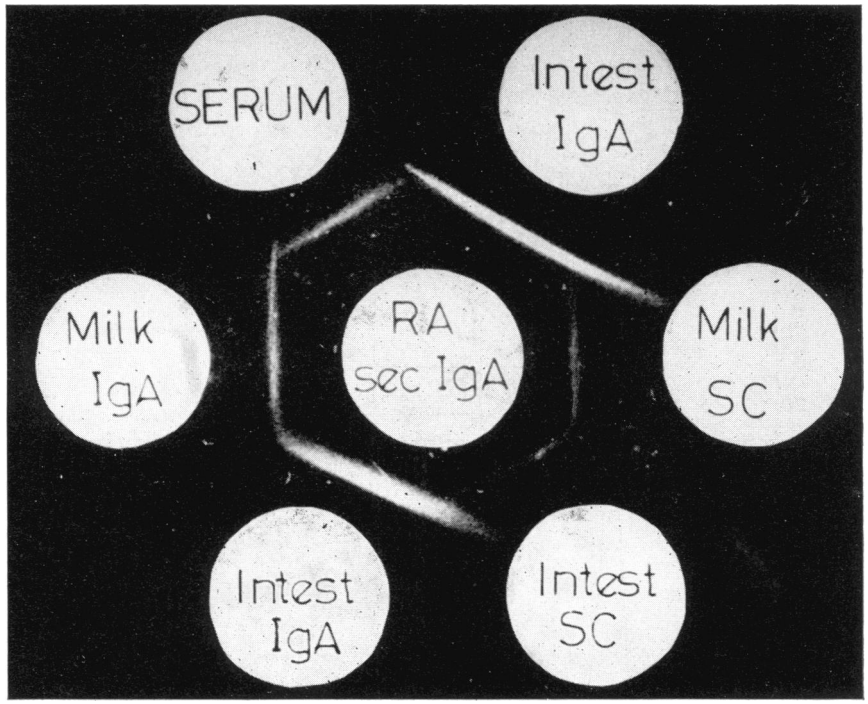


FIG. 5. Immunological double diffusion studies demonstrating secretory component and secretory IgA in intestinal secretions.

underlying lamina propria lymphoid cells containing IgA were present mainly in the region of the crypts and towards the muscularis mucosae. The cells were fairly numerous in the duodenum and jejunum but very few were seen in the lamina of the ileum (Table 2).

The localization of IgM in the duodenum, jejunum and ileum was generally similar to that described for IgA. The immunoglobulin was observed in the apical cytoplasm of the epithelial cells of the lower regions of the crypts, and generally appeared as a precisely defined narrow band in the region of the terminal web, just below the microvilli. Numerous stained cells were demonstrated in the lamina propria of the jejunum and to a lesser extent, in the duodenum, usually in the tissues surrounding the lower crypts. Only occasionally were similar cells seen in the lamina of the ileum (Table 2), but a considerable number of stained cells were seen in Peyers patches. In relation to the immunochemical findings of the lack of affinity of IgM for the mucin in small intestinal secretions it is of interest that there was no evidence of IgM in the mucin layer on the epithelial cells of the villi.

In tissue stained for IgG the immunoglobulin was seen as finely divided fluorescent particles in extravascular deposits throughout the connective tissue of the lamina propria, both in the villous cores and surrounding crypts at all levels of the gut. Stained lymphoid cells were observed to be scattered amongst the large numbers of autofluorescing (pinkish-white) eosinophilic cells of the lamina propria. These were mainly situated in the tissue surrounding the crypts. However the large amount of extravascular fluorescing immunoglobulin precluded precise cell counts being obtained.

Examples of immunofluorescent localization of immunoglobulins in intestinal tissues of the preruminant calf which show some of the observations mentioned above are given in Fig. 7. In relation to the onset and development of the synthesis of immunoglobulins in the intestine it was shown that lymphoid cells of the lymphatic tissue of the ileum from a 7-day-old calf showed evidence of all three immunoglobulins.

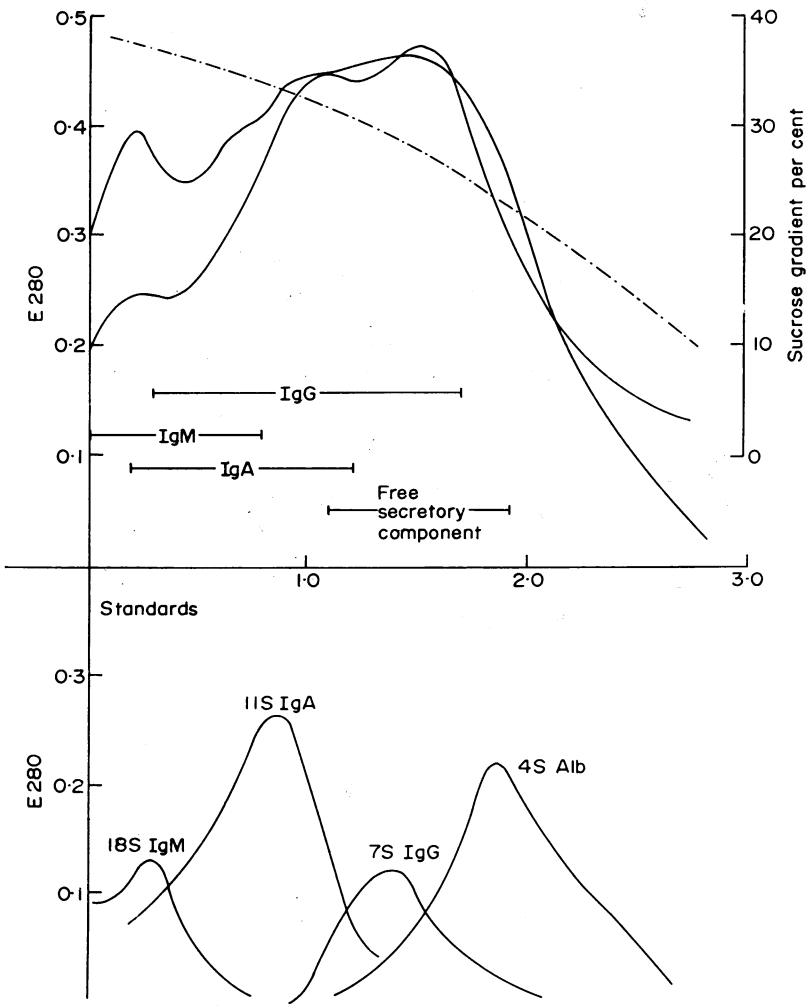


FIG. 6. Molecular size characteristics of immunoglobulins in calf intestinal secretions confirmed by density gradient ultracentrifugation.

Secretory component was demonstrated in crypt epithelial cells at all three levels of the small intestine. Fluorescent activity was precisely localized in a narrow zone of the apical cytoplasm extending from the terminal web region to the microvilli (Fig. 8). In contrast to the findings in other species, goblet cells of both crypts and villi were generally unstained; fluorescence of crypt goblet cells being observed in one animal only.

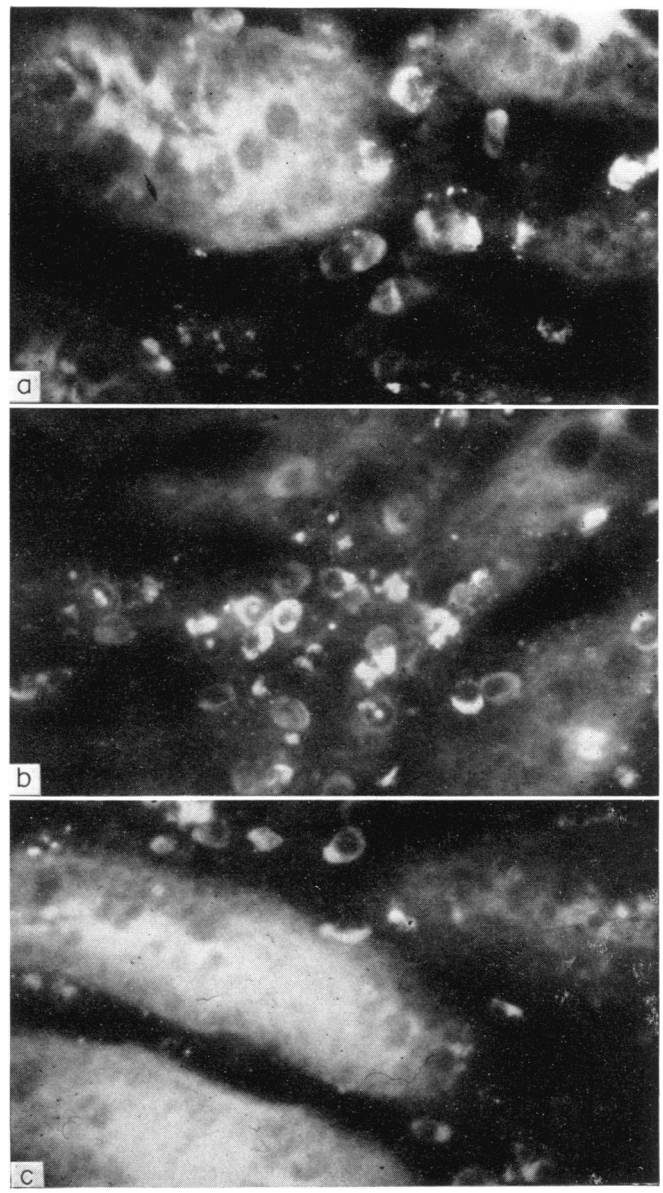


FIG. 7. Bovine jejunum stained with rabbit anti-bovine immunoglobulin FITC conjugate showing localization of immunoglobulin in the epithelial cells and surrounding lamina propria. (a and b) IgA; (c) IgM.

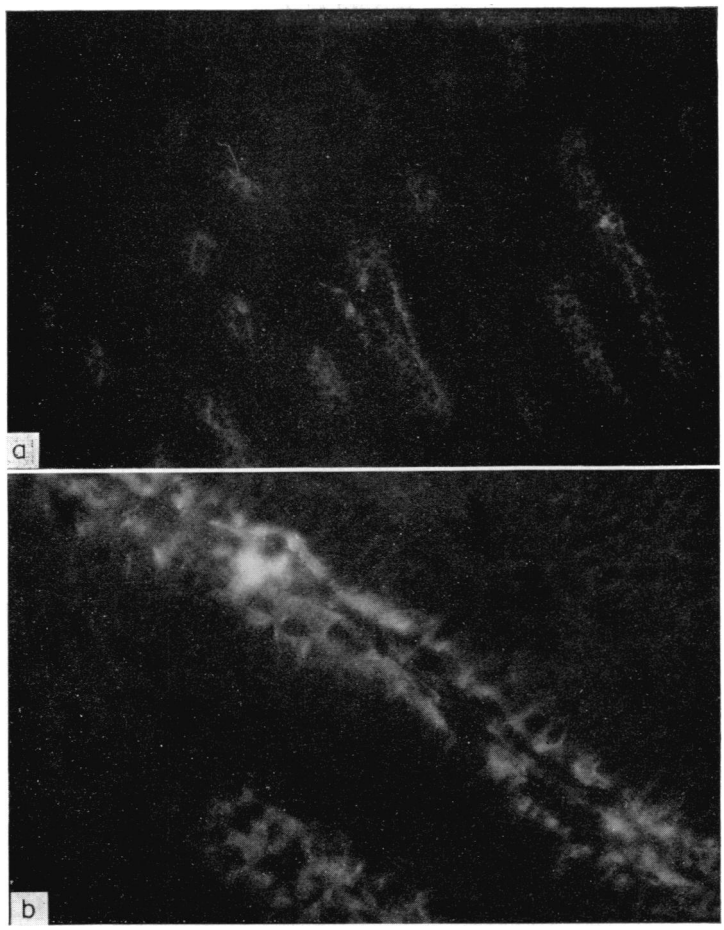


FIG. 8. Bovine jejunum stained with rabbit anti-bovine secretory component FITC conjugate showing it in the apical cytoplasm of crypt epithelium but not the cells of the lamina propria. (a) low power; (b) high power.

TABLE 1

PROTEINS AND IMMUNOGLOBULINS IN INTESTINAL SECRETIONS OF PRE-RUMINANT FISTULATED CALVES

| Calf No. | Type of fistula | Total protein (mg/100 ml) | IgG1 (mg/100 ml) | IgG2 (mg/100 ml) | IgM (mg/100 ml) | IgA (mg/100 ml) |
|---|---|---|---|---|---|---|
| 16 | Thiry-Vella jejunum | 395–1080 (783 ± 230) | 1·5–7·4 (4·6 ± 2·5) | 11·0–178·0 (95·2 ± 77·8) | 17–47 (31 ± 13·5) | 13–27 (21 ± 5·2) |
| 17 | Thiry-Vella jejunum | 670–1200 (960 ± 206) | 1·6–3·6 (2·8 ± 0·8) | 11·0–56·0 (38·0 ± 15·4) | 81–130 (104 ± 22·5) | 12–70 (42 ± 23) |
| 18 | Thiry-Vella jejunum | 1240–1920 (1676 ± 255) | 2·3–15·8 (7·6 ± 4·8) | 890–2420 (163·4 ± 68·4) | 98–120 (107 ± 9·4) | 42–96 (60 ± 14·3) |
| 19 | Double re-entrant jejunum | 560–1360 (883 ± 423) | 2·7–14·0 (9·4 ± 4·1) | 2·9–9·8 (6·3 ± 2·9) | 29–81 (56 ± 21·3) | 13–96 (56 ± 39·4) |
| 20 | Double re-entrant jejunum | 630–1300 (936 ± 246) | 1·6–2·6 (2·1 ± 0·5) | 2·9–7·8 (5·5 ± 2·1) | 21–100 (43 ± 32·8) | 12–42 (21 ± 7·8) |
| 21 | Double re-entrant jejunum | 723–1124 (963 ± 159) | 7·0–24·0 (13·4 ± 6·3) | 2·9–4·8 (3·7 ± 0·8) | 10–95 (53 ± 37·7) | 7–54 (30 ± 20·4) |

TABLE 2

DISTRIBUTION OF CELLS SYNTHESIZING IgA AND IgM IN THE LAMINA PROPRIA OF BOVINE SMALL INTESTINE

| Calf No. | Tissue | Number of cells/20 fields | |
|---|---|---|---|
| | | IgA | IgM |
| 17 | Duodenum | 1090 | 147 |
| | Jejunum | 470 | 246 |
| | Ileum | 17 | 35 |
| 18 | Jejunum | 146 | 95 |
| 19 | Duodenum | 231 | 87 |
| | Jejunum | 133 | 67 |
| | Ileum | 52 | 77 |
| 20 | Duodenum | 356 | 155 |
| | Jejunum | 484 | 215 |

## DISCUSSION

Early in the life of all herbivorous animals, a part of the alimentary tract develops with the specific purpose of providing a compartment for retention and microbial fermentation of the fibrous parts of the diet. In the bovine this specialized digestive facility is provided by a complex forestomach consisting of four compartments: rumen, reticulum, omasum and abomasum. The cellulolytic activity of the microflora in the forestomach of the ruminant provides numerous metabolites essential for the survival of the animal (Annison and Lewis, 1959). However the function of the remainder of the alimentary tract is unlikely to differ from that of the monogastric species and it therefore seems likely that the immune defence mechanism common to monogastric animals was equally effective in the calf since calves have the same need for protection against enterobacterial infections.

In view of what is known of the immunology of bovine secretions the present findings prove very interesting. Extrapolating from the observations in milk and saliva (Sullivan *et al.*, 1969) one might have anticipated that a selective transfer of IgG1 could again be the operative local immune defence system in the intestine of the calf. The present studies clearly show that this is not the case. Thus, although IgG1 is quantitatively the major immunoglobulin acquired by the calf from the colostrum, and high blood serum levels are maintained throughout early life, very little of this component appears to cross the intestinal epithelium again to contribute to external defence. On the other hand IgG2 which is almost completely excluded from secretion in the colostrum, milk and saliva and is present only in very low concentrations in post-colostral calf serum, appears in the intestinal secretions in higher concentrations than IgG1. In one calf for example IgG2 was consistently the major immunoglobulin in intestinal secretions.

The initial thesis of Heremans, Crabbe and Masson (1966) and Tomasi (1967) that IgA is probably functional in external defence by providing protection of epithelial surfaces finds increasing support in investigations on numerous species (reviewed by Tomasi and Beinenstock, 1969). Studies of immunity in the gastrointestinal tract suggest that antibody is predominantly attributable to IgA (Keller, Dwyer, Oh and Amodio, 1969; Crabbe, Nash, Bazin, Eyssen and Heremans, 1969; Ogra and Karzen, 1969). The potentially important role of intestinal IgA synthesis in host defence mechanisms against the gut microflora is further emphasized by studies in the germ free mouse (Crabbe, Bazin, Eyssen

and Heremans, 1968). It is suggested that the microflora of the intestine may be the major antigenic stimulus for development of IgA synthesizing immunocytes of the lamina propria.

The presence of a characteristic IgA system as part of the intestinal secretory immune mechanism of the calf is confirmed by the fact that the molecule has all the physiochemical properties of secretory 11S IgA described in man (Tomasi, Tan, Solomon and Prendergast, 1965; South, Cooper, Wolheim, Hong and Good, 1966). There is also evidence for free and bound secretory component. The immunofluorescent localization of IgA and secretory component in bovine intestinal tissue is also similar to that described for human (Tourville, Adler, Beinenstock and Tomasi 1969) and porcine intestine (Porter and Allen, 1972). However it is clear from the quantitative studies of secretions from jejunal loops and also from the relative counts of cells containing IgA and IgM that the latter is of considerable significance in the bovine immune response. The importance of IgM in serological defence of the calf against gram negative organisms has been stressed previously (Penhale, 1965; Klaus, Bennett and Jones, 1969).

The majority of authors have emphasized the role of IgA in external defence without reference to the fact that the functional significance of an immunoglobulin is not necessarily related to its concentration. Antibody in the intestine is not necessarily limited to IgA (Felsenfeld, Greer and Felsenfeld, 1967; Kriebal, Kraft and Rotherberg, 1969; Porter, Noakes and Allen, 1970) and it is significant that the role of IgA in external defence may be taken over by IgM in cases of IgA deficiency (Stobo and Tomasi, 1967; Eidelman and Davis, 1968). Brandtzaeg (1968) has suggested that a common secretory mechanism may operate for IgA and IgM, also Thompson (1970) has provided evidence that IgM may also complex secretory component. The presence of IgM as well as IgA in crypt epithelium has also been demonstrated previously in immunofluorescent studies of porcine intestine (Allen and Porter, 1970) showing that these two immunoglobulins may have a complementary role as antibodies in defence of the intestinal mucosa.

The apparent lack of affinity of IgM for intestinal mucous, demonstrated by the immunochemical studies of mucous gels formed in intestinal secretions and also by immunohistological observations of the intestinal epithelium, may militate against its effective antibody function. The binding of an antibody in the mucous spread over the epithelial surface might have considerable value in providing an immunological barrier in which the antibody is present in a relatively high local concentration. Other factors such as lysozyme and complement will also be required for the immunoglobulin to deal effectively with a bacterial challenge and if the processes of immune synthesis and secretion do little more than release such antibody into the lumen, then there is little possibility of an effective local defence.

Thus the physical relationships of each class of immunoglobulin with the intestinal mucous together with their relative antibody activities will have to be assessed before a reasonable prediction can be made of their protective capacities in the host defence mechanism. The complex secretion of immunoglobulins in the calf intestine presents an interesting challenge in defining the most important factors in local intestinal defence.

## ACKNOWLEDGMENT

These investigations were carried out with the technical assistance of Mrs L. Pugh and Mr M. E. Prior.

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