

## Physicochemical Characterization of Proteolytic Cleavage Fragments of Bovine Colostral Immunoglobulin G<sub>1</sub> (IgG<sub>1</sub>)

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(Received 1 August 1975)

Normal bovine colostrum immunoglobulin G<sub>1</sub> was subjected to enzymic digestion (pepsin, papain and trypsin) and the resulting fragments separated by a combination of molecular-sieve and phosphocellulose chromatography. Fragments F(ab')<sub>2</sub> derived from peptic digestion, fragment Fab from papain digestion and fragment Fab(t) from tryptic digestion showed complete antigenic identity with each other. Although fragment F(ab')<sub>2</sub> (peptic digestion) had a sedimentation coefficient (*s*<sub>20,w</sub>) of 5.3S, those for fragments Fab' (peptic digestion), Fab (papain digestion) and Fab(t) (tryptic digestion) were found to be 3.9S, 3.7S and 3.7S respectively. The mol.wts. calculated for the various fragments from the sedimentation equilibrium data were: F(ab')<sub>2</sub>, 104000 ± 200; Fab', 51900 ± 340; Fab, 50900 ± 230; Fab(t) 50900 ± 300. Fragment Fc' (peptic digestion) had an *s*<sub>20,w</sub> of 3.2S and a mol.wt. of 42900 ± 650; fragment Fc (papain digestion) had an *s*<sub>20,w</sub> of 3.7S and a mol.wt. of 50800 ± 300; fragment Fc(t) had an *s*<sub>20,w</sub> of 3.7S and a mol.wt. of 50800 ± 450.

Different classes of immunoglobulins known to be present in bovine colostrum are IgM§, IgA, IgG<sub>1</sub> and IgG<sub>2</sub> (Butler, 1969; Duncan *et al.*, 1972; Mach *et al.*, 1968; Porter, 1971; Tewari & Mukkur, 1975). However, unlike colostrum from other mammals, IgA forms only a minor constituent of bovine milk (Mach *et al.*, 1969). Colostral IgM and IgG<sub>1</sub>, on the other hand, are present in a much larger quantity as compared with IgG<sub>2</sub> (Butler, 1969; Mukkur & Froese, 1971; Tewari & Mukkur, 1975). The fact that colostrum IgG<sub>1</sub> was shown to possess a higher molecular weight than IgG<sub>2</sub> (Duncan *et al.*, 1972; Tewari & Mukkur, 1975) led to a postulation that an immunoglobulin molecule such as IgG<sub>1</sub> with a molecular weight greater than that found conventionally for mammalian IgG might act as a functional substitute for secretory IgA in milk, especially since the latter is present in very small quantities (Mach *et al.*, 1969). Secretory IgA has been found to be relatively resistant to the action of certain proteolytic enzymes such as papain, pepsin, trypsin and chymotrypsin (Ghetie & Mota, 1973; Tomasi & Bienenstock, 1968), the resistance being due to the presence of the secretory component (Ghetie & Mota, 1973). The present investigation was therefore performed to determine the susceptibility of normal bovine colostrum IgG<sub>1</sub> to the action of various proteo-

lytic enzymes including pepsin, papain and trypsin and to isolate and characterize the cleavage products.

### Materials and Methods

#### *Isolation of bovine colostrum IgG<sub>1</sub>*

Bovine colostrum IgG<sub>1</sub> was isolated as described by Tewari & Mukkur (1975). Briefly, bovine colostrum whey was prepared from colostrum as described previously (Mukkur & Froese, 1971). The immunoglobulins were precipitated with satd. (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> (final concn. 50%). After dialysis against 0.32M-NaCl/0.01M-Tris/HCl, pH8.0, the globulin fraction was subjected to gel filtration on Bio-Gel P-300 (50.0cm × 110.0cm) (Bio-Rad Laboratories, Montreal, Canada) equilibrated with 0.32M-NaCl/0.01M-Tris/HCl, pH8.0, when two major peaks were obtained. The 7S peak, representing IgG types, was subjected to ion-exchange chromatography on TEAE-cellulose (1.5cm × 30.0cm) (Bio-Rad) by using a stepwise elution. Though IgG<sub>2</sub> was eluted with 0.001M-Tris/HCl, pH8.6, IgG<sub>1</sub> was eluted with buffers consisting of 0.001M-Tris/HCl, pH8.6, plus NaCl added in various increasing quantities, namely 0.089M, 0.14M and 0.32M. Immunoglobulin G fractions eluted with these buffers were all shown to be IgG<sub>1</sub> (Tewari & Mukkur, 1975) and showed complete antigenic identity with each other on Ouchterlony (1949) plates, which were developed by using monospecific antisera prepared in our laboratory (Tewari & Mukkur, 1975). For studies reported here,

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§ Abbreviations: IgM, immunoglobulin M; IgA, immunoglobulin A; IgG<sub>1</sub>, immunoglobulin G<sub>1</sub>; IgG<sub>2</sub>, immunoglobulin G<sub>2</sub>.

all IgG<sub>1</sub> fractions were pooled and used for various enzymic digestions.

#### *Preparation of anti-(bovine colostrum IgG<sub>1</sub>) antisera*

Anti-(bovine colostrum IgG<sub>1</sub>) antisera were prepared by intradermal injection of rabbits with 1.0 ml of purified colostrum IgG<sub>1</sub> (1.0 mg/ml) in complete Freund's adjuvant, followed by subcutaneous booster injections at monthly intervals. The rabbits were bled periodically.

#### *Determination of bovine colostrum IgG<sub>1</sub> concentration*

IgG<sub>1</sub> concentration was determined by using an  $E_{280}^{1\%}$  of 12.1 (1 cm light-path) as determined previously (Tewari & Mukkur, 1975).

#### *Enzymic digestions of bovine colostrum IgG<sub>1</sub>*

(a) *Peptic digestion.* The procedures used for pepsin digestion of normal bovine colostrum IgG<sub>1</sub> were essentially those of Nisonoff *et al.* (1961). The samples, at a concentration of 50 mg/ml, were incubated with pepsin (crystallized; Worthington Biochemical Corp., Toronto, Ont., Canada) at a protein/enzyme ratio of 50:1, in 0.2 M-sodium acetate, pH 4.5, at 37°C in a water bath. The incubations were varied between 1 h and 36 h with periodic checks of pH and temperature in order to maintain constancy. The reactions were stopped by adjusting to pH 8.0 with concentrated Tris solution. The digestion products were dialysed against 0.2 M-sodium acetate, pH 7.5, and either stored frozen until used or fractionated directly by chromatography.

(b) *Papain digestion.* Papain digestions were carried out essentially as described by Porter (1959) with slight modifications. Briefly, the reaction mixtures consisted of IgG<sub>1</sub> (50 mg/ml) in 0.1 M-sodium phosphate buffer, pH 7.0, papain (1%, w/w; Dickson and Co., Orangeburg, NY, U.S.A.), 0.01 M-cysteine and 2 mM-EDTA. The enzymic digestions were carried out by incubations of the reaction mixtures at 37°C for 6, 12, 18, 24, 30 and 36 h. The reactions were stopped by alkylation with 100% molar excess of sodium iodoacetate (Sigma Chemical Co., St. Louis, MO, U.S.A.) dissolved in 0.2 M-Tris/HCl, pH 8.0. The samples were then dialysed exhaustively against 0.1 M-Na<sub>2</sub>HPO<sub>4</sub>, pH 7.0, in the cold-room (4°C). The digestion products were either frozen until ready to use or fractionated directly by chromatography.

(c) *Tryptic digestion.* Tryptic digestions were performed as described by Edelman (1967). IgG<sub>1</sub> (100 mg) dissolved in 2 ml of 0.15 M-NaCl containing 0.05 M-CaCl<sub>2</sub> was incubated in a water bath at 37°C, and the pH adjusted to 8.0. Trypsin (2%, w/w; Sigma) was added in the reaction vial, and the reaction stopped by addition of soya-bean trypsin inhibitor (Nutritional Biochemical Corp., Cleveland, OH, U.S.A.) at an enzyme/inhibitor ratio of 5:1 (w/w). The samples were chilled at 4°C and were

dialysed exhaustively against 0.1 M-Na<sub>2</sub>HPO<sub>4</sub>, pH 7.0, and stored frozen until ready for fractionation.

#### *Analytical ultracentrifugation*

A Beckman model E analytical ultracentrifuge equipped with electronic speed control was used. Sedimentation-velocity runs were made at 52000 rev./min at 20°C in an An-H rotor. The method of Schachman (1957) was used to correct  $s_{obs.}$  to  $s_{20, w.}$

Sedimentation equilibrium runs were made either at 24000 rev./min or 30000 rev./min at 20°C. Then the molecular weights were determined by the meniscus-depletion method of Yphantis (1964). Partial specific volumes ( $\bar{v}$ ) of the digestion fragments were calculated by the H<sub>2</sub>O-<sup>2</sup>H<sub>2</sub>O method of Edelstein & Schachman (1967).

#### *Immunological tests*

The purity of isolated immunoglobulins and different enzymic-digestion fragments were tested by immunoelectrophoresis as described by Scheidegger (1955), and Ouchterlony's (1949) gel-diffusion technique. The latter was also used to determine the antigenic relationships between fragments obtained from the various different enzymic digestions.

#### *Reduction and alkylation*

Reduction of purified fragment F(ab')<sub>2</sub> obtained from peptic digestion of IgG<sub>1</sub> was carried out in 0.1 M-Na<sub>2</sub>HPO<sub>4</sub>, pH 7.0, by using 0.3 M-cysteine for 12 h under an N<sub>2</sub> atmosphere. Alkylation was performed by addition of a 100% molar excess of sodium iodoacetate, pH 7.0.

#### *Agglutination tests*

The agglutination tests were performed with IgG<sub>1</sub> isolated from the colostrum of cows immunized with a *Staphylococcus aureus* vaccine as described by Mukkur & Tewari (1975), its peptic fragment F(ab')<sub>2</sub> and Fab', which was prepared by reduction of F(ab')<sub>2</sub> as described above. The agglutination tests were performed using a thick live suspension of *S. aureus* as antigen (10<sup>9</sup> bacteria/ml). Serial twofold dilutions of the above samples were made in 1% inactivated normal rabbit serum in 0.85% NaCl as diluent. A sample (0.5 ml) of each of the serial dilutions was mixed with 0.5 ml of the bacterial suspension. The control contained only the diluent and the bacterial suspension. The test tubes were incubated for 2 h at 37°C in a water bath followed by an 18 h incubation at room temperature (23°C). An agglutination test was recorded as positive if bacteria were uniformly spread at the bottom of the test tube, with or without serrated edges, and the supernatant was transparent. If the supernatant was partially turbid the test was considered doubtful; if the bacteria settled in the form of a button with a clear or translucent super-

nant, the agglutination test was recorded as negative.

## Results and Discussion

### *Investigations of the aggregation behaviour of normal bovine colostrals IgG<sub>1</sub> in different buffers*

It was reported previously that both bovine colostrals and serum IgG<sub>1</sub> aggregated in certain buffers, but not in others (Tewari & Mukkur, 1975). Therefore, in order to carry out the enzymic digestion of IgG<sub>1</sub> in an unaggregated state, it was necessary to ascertain that IgG<sub>1</sub> did not aggregate in buffers used in different types of enzymic digestions. To this end, sedimentation-velocity runs of different concentrations of IgG<sub>1</sub> (2.0–10.0 mg/ml) were made in 0.2M-sodium acetate, pH 4.5 (for peptic digestion), 0.1M-Na<sub>2</sub>HPO<sub>4</sub>, pH 7.0 (for papain digestion), and 0.15M-NaCl/0.05M-CaCl<sub>2</sub>, pH 8.0 (for tryptic digestion). That no aggregation of IgG<sub>1</sub> occurred in these media was evident from the fact that IgG<sub>1</sub> showed a negative concentration dependence of sedimentation coefficients in these media.

#### (a) Peptic digestion

*Hydrolysis of IgG<sub>1</sub> by pepsin.* The pepsin digested mixtures from different incubation periods as described in the Materials and Methods section, were dialysed against 0.2M-sodium acetate, pH 8.0, at 4°C. On analytical ultracentrifugation, only one peak with an  $s_{20,w}$  of 5.3S was observed, regardless of the time of digestion, even though fragment Fc' constituted about 25% of the total digest. This observation could be explained by the presence of non-covalent interactions between fragment F(ab')<sub>2</sub> and Fc', and is supported, as described later, by our inability to resolve these fragments on molecular sieve chromatography. As IgG<sub>1</sub> has an  $s_{20,w}$  of 7.2S, it was clear that the protein had been split into a large fragment(s) and possibly small peptide fragment(s).

Immunoelectrophoretic patterns of various peptic digests showed the presence of three components. One component migrated toward the anode and another toward the cathode. The third component, which appeared as a faint line, was found to be undigested IgG<sub>1</sub> in each of these digestion mixtures, and migrated toward the cathode like IgG<sub>1</sub>.

*Isolation of fragments hydrolysed by pepsin.* A portion (200 mg) of a 2 h peptic digest was dialysed against 0.32M-NaCl/0.01M-Tris/HCl, pH 8.0, and filtered through a Bio-Gel P-200 column (2.5 cm × 100.0 cm) equilibrated with the same buffer, and two major peaks were obtained (Fig. 1a). The first fraction (peak 1) was composed of undigested IgG<sub>1</sub> as determined by complete antigenic identity with untreated IgG<sub>1</sub>, whereas the second fraction (peak 2)

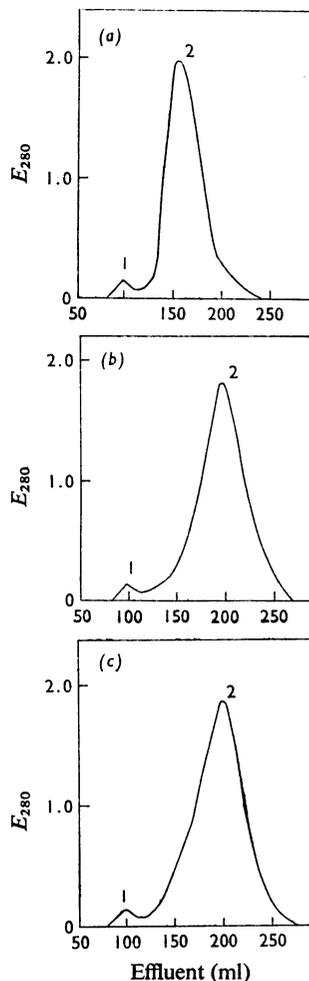


Fig. 1. Elution profiles of proteolytic digest of bovine colostrals IgG<sub>1</sub>

A portion of bovine colostrals IgG<sub>1</sub> was digested with pepsin (2 h), papain (12 h) and trypsin (2 h) and subjected to gel filtration on a Bio-Gel P-200 column (2.5 cm × 100.0 cm) equilibrated with 0.32M-NaCl/0.01M-Tris/HCl, pH 8.0. Profiles show: (a) pepsin digest; (b) papain digest; (c) trypsin digest.

was found to be composed of the mixtures of F(ab')<sub>2</sub> and possibly Fc' fragments as described below.

Attempts to separate F(ab')<sub>2</sub> from Fc' fragments by methods described previously for mammalian systems such as gel-filtration on Sephadex G-75 (Utsumi & Karush, 1965) and DEAE-cellulose chromatography (Porter, 1959) were not successful. Peak 2 (Fig. 1) was concentrated by pervaporation, dialysed against 0.025M-Na<sub>2</sub>HPO<sub>4</sub>/0.3M-NaCl, pH 4.2, and subjected to ion-exchange chromatography on a phosphocellulose column (2.5 cm × 35.0 cm) by using discontinuous gradient elution. The first

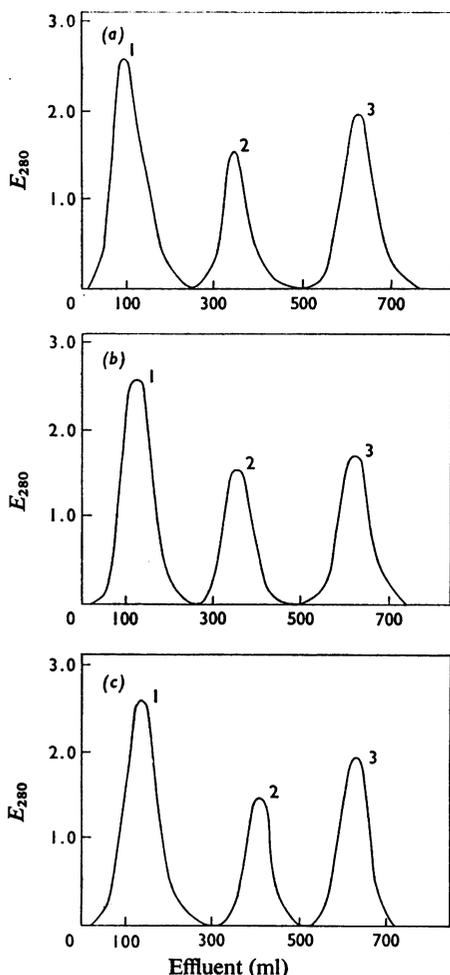


Fig. 2. Phosphocellulose chromatography of fraction 2 obtained by gel filtration of various enzymic digests as shown in Plate 2

Fraction 2 was obtained from the peptic digest and subjected to ion-exchange chromatography on phosphocellulose (2.5 cm  $\times$  30.0 cm) by using a stepwise gradient elution. The solvents used were: (1) 0.025 M-Na<sub>2</sub>HPO<sub>4</sub>/0.3 M-NaCl, pH 4.2; (2) 0.025 M-Na<sub>2</sub>HPO<sub>4</sub>/0.5 M-NaCl, pH 4.2; (3) 0.1 M-Na<sub>2</sub>HPO<sub>4</sub>, pH 7.4. Profiles shown: (a) pepsin digest; (b) papain digest; (c) trypsin digest.

solvent used for elution was 0.025 M-Na<sub>2</sub>HPO<sub>4</sub>/0.3 M-NaCl, pH 4.2 (300 ml), followed by 0.025 M-Na<sub>2</sub>HPO<sub>4</sub>/0.5 M-NaCl, pH 4.2 (300 ml), as the second solvent. The third solvent used was 0.1 M-Na<sub>2</sub>HPO<sub>4</sub>, pH 7.4 (300 ml). Three peaks, 1, 2 and 3, were obtained (Fig. 2a). Yields of these three fractions were calculated by summing the  $E_{280}$  in each fraction. The ratio of the yields for the three fractions (1:2:3) was 1.0:0.4:0.5 and the total recovery from the column was 70–74%.

On Ouchterlony (1949) gel-diffusion tests, fractions 1 and 2 were found to be antigenically identical to each other, and therefore were pooled together. Fractions 3 and 1, and 3 and 2, on the other hand, showed non-identity. However, a partial identity was observed between fraction 2 and IgG<sub>1</sub>, and fraction 3 and IgG<sub>1</sub> (results not shown). The antiserum used for development was rabbit anti-(colostral IgG<sub>1</sub>) antiserum. To find out the localization of antibody activity in these fragments, fractions 1, 2 and 3 were prepared by using colostral IgG<sub>1</sub> possessing anti-(*S. aureus*) activity. Although IgG<sub>1</sub> and pooled fractions 1 and 2 agglutinated *S. aureus*, fraction 3 did not. Therefore fractions 1 and 2 corresponded to the antigen binding fragment F(ab')<sub>2</sub>, as reported by Nisonoff *et al.* (1961) in the rabbit system, whereas fraction 3 corresponded to the Fc' fragment.

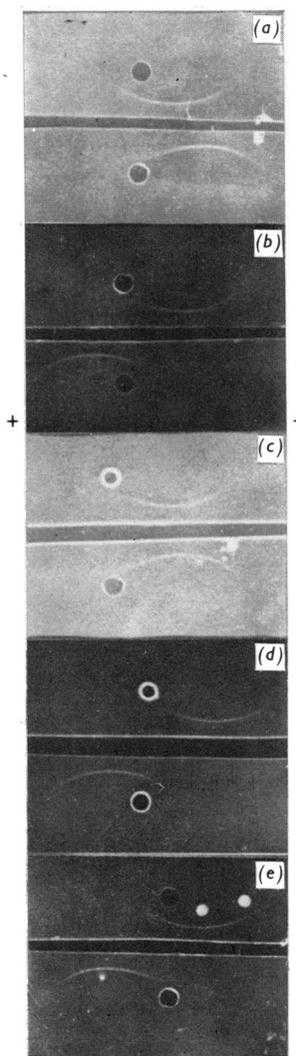
Immunoelectrophoretic patterns of IgG<sub>1</sub>, F(ab')<sub>2</sub> and Fc' fragments showed that the first two migrated toward the cathode whereas Fc' fragment was composed of an anodally migrating component (Plate 1a and 1b).

#### Conversion of fragment F(ab')<sub>2</sub> to Fab'

Reduction and alkylation of fragment F(ab')<sub>2</sub> was carried out as described in the Materials and Methods section. The reaction mixture was dialysed against 0.1 M-Na<sub>2</sub>HPO<sub>4</sub>, pH 8.0, and filtered through a Bio-Gel P-60 column (1.5 cm  $\times$  30.0 cm) equilibrated with the same medium. Two peaks were obtained. About 60% of the original F(ab')<sub>2</sub> ( $V_e/V_0 = 2.0$ ) was reduced to Fab', which was eluted from the column in the second peak ( $V_e/V_0 = 4.0$ ).

Immunoelectrophoretic analysis revealed that both fragments F(ab')<sub>2</sub> and Fab' migrated toward the cathode (Plate 1c). In addition, fragment F(ab')<sub>2</sub> and Fab' were found to be antigenically identical as judged by Ouchterlony's (1949) gel-diffusion technique (results not shown). Agglutination tests conducted with fragment Fab' showed that it did not agglutinate a *S. aureus* suspension, thus attesting to its already known monovalency.

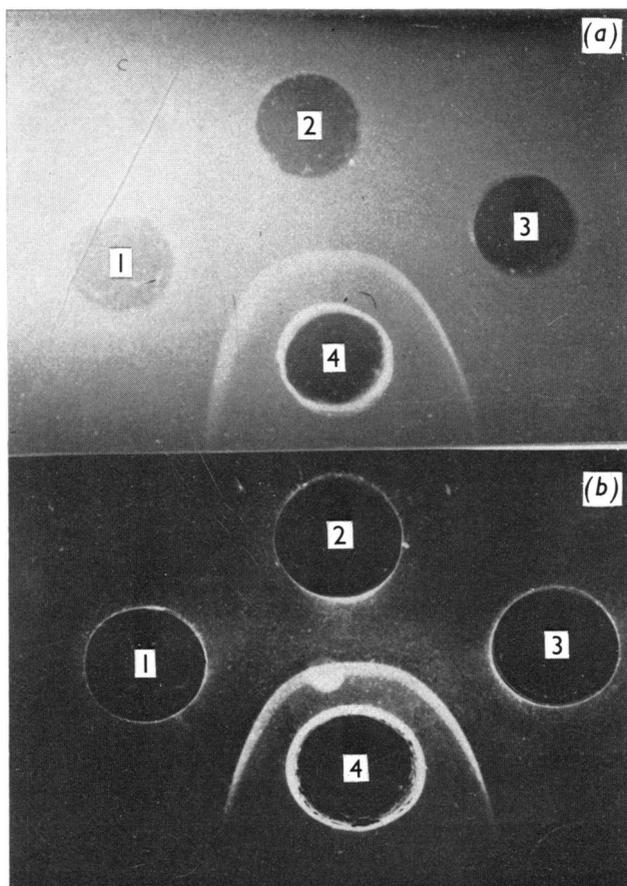
**Molecular weight and sedimentation coefficient ( $s_{20,w}$ ).** Analytical ultracentrifugation of F(ab')<sub>2</sub>, Fab' and Fc' revealed the  $s_{20,w}$  values of 5.3, 3.9 and 3.2S respectively (Table 1). Although the partial specific volumes ( $\bar{v}$ ) of fragment F(ab')<sub>2</sub> and Fc' were calculated to be 0.768 and 0.753 ml/mg respectively, that used for molecular weight determination of fragments Fab' was assumed to be the same as that of fragment F(ab')<sub>2</sub>. The mol. wts. thus calculated were 104000  $\pm$  200, 51900  $\pm$  340 and 42900  $\pm$  650 for fragments F(ab')<sub>2</sub>, Fab' and Fc' respectively (Table 1). The nature of association of the fragment Fc', whether covalent or non-covalent, was not determined in this study. One of the most interesting findings in our system was that unlike the rabbit (Nisonoff *et al.*, 1960) and the human system (Heimer



EXPLANATION OF PLATE I

*Immunoelectrophoretic patterns of IgG<sub>1</sub> and various enzymic fragments*

(a) IgG<sub>1</sub> (top) and F(ab')<sub>2</sub> (bottom); (b) F(ab')<sub>2</sub> (top) and Fc' (bottom); (c) F(ab')<sub>2</sub> (top) and Fab' (bottom); (d) Fab (top) and Fc (bottom) produced by papain digestion; (e) Fab(t) and Fc(t). The central troughs contained rabbit anti-(bovine colostrum IgG<sub>1</sub>) antisera.



EXPLANATION OF PLATE 2

*Ouchterlony analysis showing antigenic relationship between enzymically produced fragments*

(a) Fab' (well 1), Fab (well 2) and Fab(t) (well 3). Ouchterlony (1949) plates were developed with rabbit anti-(bovine colostral IgG<sub>1</sub>) antisera (well 4). (b) Fc' (well 1), Fc (well 2) and Fc(t) (well 3). Ouchterlony plates were developed with specific rabbit anti-(bovine colostral IgG<sub>1</sub>) antisera (well 4).

**Table 1.** *Partial specific volumes,  $s_{20,w}$  and molecular weights of normal bovine colostrals IgG<sub>1</sub> and its peptic, papain and tryptic fragments*

The concentration of IgG<sub>1</sub> and its various fragments used for analytical ultracentrifugation to determine  $s_{20,w}$  was 5 mg/ml.

	Enzyme used for digestion	$s_{20,w}$ (S)	Partial specific volume	Molecular weight
IgG <sub>1</sub>	—	7.2*	0.726	156000†
F(ab') <sub>2</sub>	Pepsin	5.3	0.768	104000 ± 200
Fab'	Pepsin	3.9	0.768‡	51900 ± 340
Fc'	Pepsin	3.2	0.753	42900 ± 650
Fab	Papain	3.7	0.768‡	50900 ± 230
Fc	Papain	3.7	0.753§	50800 ± 300
Fab(t)	Trypsin	3.7	0.768‡	50900 ± 300
Fc(t)	Trypsin	3.7	0.753§	50800 ± 450

\*  $s_{20,w}^0$ .

† Determined by Tewari & Mukkur (1975).

‡ Assumed to be the same as that for F(ab')<sub>2</sub>.

§ Assumed to be the same as that for Fc'.

*et al.*, 1969), fragment Fc' was not completely digested regardless of the time of digestion, therefore suggesting a possible conformational difference between the Fc region(s) of bovine colostrals IgG<sub>1</sub> and other species studied so far.

**(b) Papain digestion**

*Hydrolysis of IgG<sub>1</sub> by papain.* Five digestion mixtures from different incubation periods, prepared as described previously, were dialysed exhaustively against 0.1M-Na<sub>2</sub>HPO<sub>4</sub>, pH7.0, in the cold-room (4°). This medium was used, since IgG<sub>1</sub> was found not to undergo aggregation. When these digests were examined in the analytical ultracentrifuge, only one peak, with an  $s_{20,w}$  of 3.7S, was obtained. Therefore it was clear that IgG<sub>1</sub> had been split into a large fragment or fragments of similar size with possibly some diffusible peptides.

Immunoelectrophoretic patterns of the digestion mixtures showed that three components were present, one migrating towards the anode and another towards the cathode. The third component, which appeared as a faint line, migrated towards the cathode like IgG<sub>1</sub> and was identified to be so by Ouchterlony (1949) analysis (results not shown).

*Resolution of fragments hydrolyzed by papain.* Attempts to fractionate this mixture by chromatography on CM-cellulose (Porter, 1959), Sephadex G-75 (Utsumi & Karush, 1965) and DEAE-cellulose (Porter, 1959) were not successful. However, resolution could be achieved by chromatography on phosphocellulose.

A portion (200mg) of IgG<sub>1</sub> digested for 12h in 0.1M-Na<sub>2</sub>HPO<sub>4</sub>, pH7.0, was loaded on a Bio-Gel P-200 column (2.5cm × 9.00cm) equilibrated with the same medium. Approximately 3% of the reaction

material that was eluted off the column first was found to be undigested IgG<sub>1</sub> (Fig. 1b) as judged by its complete antigenic identity with intact IgG<sub>1</sub>. The second peak was dialysed against 0.025M-Na<sub>2</sub>HPO<sub>4</sub>/0.3M-NaCl, pH4.2, and subjected to ion-exchange chromatography on a phosphocellulose column (2.5cm × 35.0cm) by using a stepwise gradient. The solvents used for elution were the same as described for pepsin digestion. Again three peaks were eluted, which were termed 1, 2 and 3, in order of elution from the column (Fig. 2b). Yields of these three fractions were measured by summing the  $E_{280}$  of each fraction. The ratio of the yields for the three fractions (1:2:3) was 1.0:0.6:0.8, and the total recovery from the column was 85–90%. Fractions 1 and 2 were antigenically identical and were therefore pooled together. These fractions were found to show a complete identity with fragment Fab' produced by reduction of peptic fragment F(ab')<sub>2</sub> and were therefore considered to constitute the Fab fragment. However, complete nonidentity was observed between Fab and Fc (fraction 3) fragments. As with the peptic products, partial identity was observed between fragments Fab and IgG<sub>1</sub>, and Fc and IgG<sub>1</sub> (results not shown). Immunoelectrophoretically, fragment Fab migrated towards the cathode, whereas Fc fragment migrated towards the anode (Plate 1d).

*Determinations of  $s_{20,w}$  and molecular weights of papain fragments.* The sedimentation coefficient ( $s_{20,w}$ ) of fragments Fab and Fc determined in 0.1M-Na<sub>2</sub>HPO<sub>4</sub>, pH7.0, were found to be 3.7S for each of the fragments (Table 1). Assuming the partial specific volumes ( $\bar{v}$ ) for fragments Fab and Fc to be the same as those calculated for pepsin-derived fragments i.e. 0.768ml/mg for Fab and 0.753ml/mg for Fc, the mol.wts. were calculated to be 50900 ± 230 and 50800 ± 300 respectively (Table 1).

**(c) Tryptic digestion**

*Hydrolysis of IgG<sub>1</sub> by trypsin.* Seven digestion mixtures from different incubation periods, prepared as described above, were dialysed exhaustively against 0.1M-Na<sub>2</sub>HPO<sub>4</sub>, pH7.0, in the cold-room. This medium was used since IgG<sub>1</sub> was found not to undergo aggregation in it. When these digests (5mg/ml) were examined in the analytical ultracentrifuge, only one peak with an  $s_{20,w}$  of 3.7S was observed. Therefore it was evident that IgG<sub>1</sub> had been split into a large fragment or fragments of similar size with possibly some diffusible peptides. As with papain digestion, immunoelectrophoretic patterns of the digestion mixtures revealed the presence of three components. One component migrated towards the anode, another towards the cathode and the third component, which appeared as a faint line and was identified as undigested IgG<sub>1</sub>, migrated towards the cathode like intact IgG<sub>1</sub>.

*Resolution of fragments hydrolysed by trypsin.* A portion (200mg) of IgG<sub>1</sub> digested for 2h in 0.015M-NaCl/0.05M-CaCl<sub>2</sub>, pH8.0, was dialysed against 0.1M-Na<sub>2</sub>HPO<sub>4</sub>, pH7.0, and loaded on a Bio-Gel P-200 column (2.5cm×90.0cm) equilibrated with the same medium. Approx. 4% of the reaction material that was eluted off the column first (Fig. 1c) was found to be undigested IgG<sub>1</sub>, as judged by its complete antigenic identity with intact IgG<sub>1</sub> (results not shown). The second peak was dialysed against 0.025M-Na<sub>2</sub>HPO<sub>4</sub>/0.3M-NaCl, pH4.2, and subjected to ion-exchange chromatography on phosphocellulose column (2.5cm×35.0cm) by using a stepwise gradient and the same solvents as described for pepsin and papain. Again three peaks were eluted (Fig. 2c), which were termed 1, 2 and 3, in order of their elution from the column. The ratio of the yields of these three fractions (1:2:3) as measured by summing the E<sub>280</sub> was 1.0:0.5:0.9, and the total recovery from the column was 87–91%. Fractions 1 and 2 were antigenically identical and were pooled together. These fractions were found to show a complete antigenic identity with the peptic Fab' and the papain-produced Fab fragments. They were therefore considered to constitute the Fab(t) fragment. As with peptic and papain fragments, Ouchterlony (1949) analysis revealed non-identity between Fab(t) and Fc(t) (fraction 3) fragments, whereas partial identity was shown between Fab(t) and IgG<sub>1</sub>, and Fc(t) and IgG<sub>1</sub> (results not shown).

Immunoelectrophoretic patterns showed that fragment Fab(t) migrated towards the cathode whereas fragment Fc(t) migrated towards the anode (Plate 1e).

*Determinations of s<sub>20,w</sub> and molecular weights of fragments Fab(t) and Fc(t).* The sedimentation coefficient (s<sub>20,w</sub>) of fragments Fab(t) and Fc(t) were determined in 0.1M-Na<sub>2</sub>HPO<sub>4</sub>, pH7.0, at a concentration of 5mg/ml and were 3.7S for each of the fragments (Table 1). The determinations of the molecular weights of fragments Fab(t) and Fc(t) were carried out by sedimentation-equilibrium analysis by assuming the partial specific volumes ( $\bar{v}$ ) to be the same as those for peptic fragments F(ab')<sub>2</sub> and Fc' prepared by pepsin digestion, i.e. 0.768ml/mg for Fab(t) and 0.753ml/mg for Fc(t), and were 50900±300 and 50800±450 respectively.

*Antigenic relationship of fragments obtained from different enzymic digestions.* Ouchterlony's (1949) gel-diffusion technique was used to test the antigenic relationship between fragments F(ab')<sub>2</sub> and Fab' (peptic digestion), Fab (papain digestion) and Fab(t) (tryptic digestion) by using rabbit anti-(bovine colostrum IgG<sub>1</sub>) antisera. A complete identity was observed between fragments F(ab')<sub>2</sub>, Fab and Fab(t) (Plate 2a). Similarly, Fc' (peptic digestion), Fc (papain digestion) and Fc(t) (tryptic digestion) were also found to antigenically identical (Plate 2b). The

antisera used for development were specific rabbit anti-(bovine colostrum IgG<sub>1</sub>) antisera. However, no precipitin lines were observed between fragments Fc', Fc and Fc(t), if specific rabbit anti-(bovine colostrum IgM) antisera were used, although fragments F(ab')<sub>2</sub>, Fab', Fab and Fab(t) again showed lines of complete antigenic identity (results not shown) thus pointing out the absence of light chain determinants on fragments Fc', Fc and Fc(t).

Earlier, Kickhöfen *et al.* (1968) had suggested that a high-molecular-weight secretory immunoglobulin G found in bovine colostrum ( $\gamma$ G<sub>s</sub>) [later identified as IgG<sub>1</sub> (Duncan *et al.*, 1972; Tewari & Mukkur, 1975)] might be a functional counterpart of IgA, which is found in very small quantities in bovine colostrum (Mach *et al.*, 1969). However, this investigation revealed that colostrum IgG<sub>1</sub> seemed, at least, structurally different from secretory IgA, since the latter is known to be relatively resistant to the action of proteolytic enzymes such as papain, chymotrypsin and trypsin (Ghetie & Mota, 1973; Tomasi & Bienenstock, 1968).

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