Electrophoretic, Immunoelectrophoretic, and Ultracentrifugal Characterization of Proteins in Whey Fractions Prepared by Salt Fractionation

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Abstract
Polyacrylamide gel disc electrophoresis (PAGE) in alkaline and acid buffers, immunoelectrophoresis, analytical ultracentrifugation, and sucrose density gradient were used to isolate immunoglobulins from bovine milk and colostrum by ammonium sulfate fractionation. The immunoglobulins were relatively free of other whey proteins and moved as one component on PAGE at pH 9.5. However, on PAGE at pH 4.3, one major and two minor components were evident. By analytical ultracentrifugation, immunoglobulins from colostrum of one cow resolved into three peaks with coefficients of 6.4S, 10.6S, and 18.8S. For a second cow, only two peaks at 6.5S and 19.0S were present. Components corresponding to these coefficients were separated by linear density gradient in 10 to 40% sucrose.

Introduction
For sometime, the salt fractionation procedure suggested by Smith has been used for the isolation of immunoglobulins from bovine milk and their subsequent separation into pseudoglobulins and euglobulins. As our knowledge of the immunoglobulins has increased, the World Health Organization (13) has proposed a more definitive nomenclature for human immunoglobulins. The Milk Protein Nomenclature Committee of the American Dairy Science Association has recommended adoption of the WHO terminology (10).

Salt fractionation alone or in combination with other techniques (3) continues to be used for separation of immunoglobulins from bovine milk or colostrum whey. This is because there is no easy, time-saving method for the preparation of workable quantities of immunoglobulins from bovine milk or colostrum.

This investigation was conducted to determine effectiveness of Smith’s procedure for the isolation of immunoglobulins in light of current knowledge and for an understanding of research in which this technique was utilized.

Experimental Procedures
Whey preparation. Fresh colostrum from an individual cow or raw mixed herd milk was obtained from the Ohio State University dairy farm. Fat was removed by centrifugation (2 to 4 C) at 2,000 x g for 15 min. Skimmilk or defatted colostrum was adjusted to pH 4.6 with 10% acetic acid and 0.1 M sodium acetate. The sample was re-centrifuged at 1,000 x g for 15 min. Precipitated caseins were discarded. Whey was adjusted to pH 6.5 with 0.5 N NaOH and the fine precipitate was removed by centrifugation at 2,000 x g for 20 min.

Immunoglobulin isolation. Neutralized whey was subjected to the salt precipitation method of Smith (11) and Fractions D, E, and F were separated. Fractions E and F were further resolved into pseudoglobulins and euglobulins. The supernatant from Fraction F was saturated with ammonium sulfate to obtain a precipitate which was designated Fraction G.

Most protein fractions were dialyzed by ultrafiltration using an Amicon Corporation (Lexington, Mass.) Diaflo apparatus with a UM-10 membrane. Samples were lyophilized and stored at -5 C.

Immunoelectrophoresis. The procedure outlined by Arquembourg et al. (1) was followed using Panagar I. D. agar (Colab, Glenwood, Illinois) and electrophoresis for 60 min at 30 mamp in .05 M barbitone acetate buffer, pH 8.6. Antisera against bovine blood serum and bovine blood gamma globulins prepared in rabbits were purchased commercially. In some experiments, antisera furnished by John Butler, USDA (3) was used.

Electrophoresis. Polyacrylamide gel disc electrophoresis in alkaline pH was by the procedure of Davis (5) with Tris-glycine buffer, pH 8.9 and a gel strength of 7.5%.

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acidity, the procedure of Reisfeld et al. (9) was followed using pH 4.5 buffer (glacial acetic acid, 8.0 ml; β-alanine, 31.2 g; and water to 1 liter) and 7.5% gel strength.

**Sedimentation.** A Beckman Model E analytical ultracentrifuge was used to determine sedimentation velocity. Sedimentations were at 20 °C and 116,272 x g (40,000 rpm) in .1 M Tris-HCl buffer, pH 8.0, containing .15 M NaCl. Wherever possible $S_0$ was determined by extrapolation to zero concentration.

**Sucrose gradient separation.** Fraction D was dissolved in .15 M NaCl. Sucrose solutions of 10, 20, 30, and 40% (w/w) were prepared in .15 M NaCl. A sucrose gradient was formed in cellulose nitrate tubes (Beckman 303934) as suggested by Gough (8). Just before centrifugation, .5 ml of the Fraction D solution was layered on the surface of each tube. The tubes were then centrifuged in a Beckman Model L preparatory ultracentrifuge at 78,000 x g for 24 hr using a type 50 angle rotor. Upon completion of centrifugation, four 2-ml portions and a final 1.3 ml portion were withdrawn from the sucrose tubes. The precipitate from the sixth fraction was dissolved in .15 M NaCl. All fractions were then dialyzed against running tap water and stored in a lyophilized state at 2 to 4 °C. For sedimentations and immunoelectrophoreses, fractions were reconstituted in .1 M Tris-HCl buffer, pH 8.0, containing .15 M NaCl.

**Results and Discussion**

Smith's fractionation procedure (11) was evaluated by polyacrylamide gel disc electrophoresis (PAGE) in alkaline and acid pH, immunoelectrophoresis (IE), and analytical ultracentrifugation in the light of current knowledge of immunoglobulins and their classes and sub-classes.

The first step in Smith's procedure involves adjustment of the pH of skimmilk to 4.5 with acid followed by centrifugation to remove precipitated caseins. The pH of the supernatant acid whey is then brought to 6.5 with NaOH. The PAGE patterns of the whey from mixed herd raw milk are in Figure 1.

Tubes A and C are PAGE electrophero-
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FIG. 2. Polyacrylamide gel disc electropherograms of: (A), neutral whey; (B) Fraction A; (C) supernetant from Fraction A; (D) Fraction C; (E) supernetant from Fraction C; (F) Fraction D; and (G) supernetant from Fraction D. Run at pH 9.5; 4 mamp/tube for 40 min.; and gel strength of 7.5%.

grams of acid whey and neutral whey, whereas Tube B was the precipitate following adjustment of the pH from 4.6 to 6.5. The pH adjustment resulted in a slight loss of whey proteins. Some loss of immunoglobulins occurred with colostrum whey.

An examination of various Smith's Fractions by PAGE is in Figure 2. Smith's Fraction A (Tube B) was obtained as a precipitate at 50% saturation with ammonium sulfate from the neutral whey. This fraction contained all of the immunoglobulins plus other milk proteins including residual caseins. The supernetant of this fraction (Tube C) was predominantly β-lactoglobulin, α-lactalbumin, and blood serum albumin with mobilities corresponding to those reported previously (4). Residual caseins were removed from Fraction A by adjustment to pH 4.6 and 25% saturation with ammonium sulfate. The precipitate formed constituted Smith's Fraction C and is shown in Tube D. The supernetant (Tube E) from Fraction C contained the immunoglobulins, proteose-peptone, and small amounts of blood serum albumin and α-lactalbumin. At this point, the supernetant was adjusted to pH 6.0 and 40% saturation with ammonium sulfate. The precipitate formed constituted Smith's Fraction D and contained immunoglobulins apparently free of other whey proteins as evidenced in the PAGE patterns in Tube F.

The separation of Fraction D on PAGE in an acid system was also undertaken. The pat-
Fig. 4. Immunoelectropherograms of Smith's Fractions. Slide A, Fraction D versus anti-bovine serum (ABS); Slide B, pseudoglobulin versus anti-bovine gamma globulins (AGG); Slide C, euglobulin versus AGG; and Slide D, Fraction G versus AGG.

Fig. 5. Immunoelectropherograms of Fraction D from two different cows (Slides A and B), euglobulin (Slide C), and pseudoglobulin (Slide D). In all cases, the upper trough contained antisera polyvalent for bovine immunoglobulins as supplied by J. E. Butler, USDA (3) and the lower trough a similar antisera but purchased commercially.
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In Slide A, IgG, IgM, and IgA may be noted in Fraction D from colostrum of a single cow when run against the USDA antisera but not when developed with commercial antisera.

For a second animal (Slide B), only IgG and IgM were evident in Fraction D with USDA antisera and only IgG with the commercial antisera. For the euglobulins (Slide C) and pseudoglobulins (Slide D), similar results were noted excepting that for the pseudoglobulins, the arc for IgM is faint. These results point out that the potency of the antisera is an important consideration in interpretation of immunological data.

In Figure 6 are schlieren patterns obtained by ultracentrifugation of Fraction D from colostrum of two different cows. Fraction D from cow A showed three distinct peaks corresponding to 6.4S, 10.6S, and 18.8S, approximating those for IgG, IgA, and IgM, respectively. Schlieren patterns of Fraction D from cow B, however, demonstrated only two peaks and these corresponded to IgG and IgM. These observations further confirm our belief that bovine milk or colostrum is either lacking or contains only very small amounts of IgA.

For density gradient separation of the immunoglobulins in a sucrose system, Fraction D obtained from Cow B was used. Five fractions were withdrawn and were examined by IE and analytical ultracentrifugation.

With respect to distribution of immunoglobulins within the sucrose gradient system, the center layers showed apparent sedimentation coefficients of 5.5 to 6.7 and IE patterns corresponding to IgG, whereas the lower-
most portion contained both IgG and IgM. When Fraction D in protein concentrations greater than 2.5% was used, a precipitate was formed in the gradient tube. The IE pattern of the resuspended precipitate (Fig. 7, A) showed a single arc against anti-gamma globulins and the schlieren pattern (Fig. 7, B) had a single peak with a sedimentation coefficient of 19.1S. Under similar experimental conditions but with 1.0 to 2.5% protein, no precipitate was evident and the major portion of IgM was located in the bottom section of the gradient tube.

It is evident that Smith's procedure (11) provides a simple approach to isolation of crude immunoglobulins from bovine milk or colostrum. By carrying the procedure only to Fraction D and with the utilization of ultrafiltration dialysis, immunoglobulins relatively free of other milk serum proteins can be isolated within a short time.

The immunoglobulins encountered in Fraction D and in subsequent fractions were heterogeneous individual classes and subclasses. Thus, a different approach for the separation of the immunoglobulin classes from Fraction D is needed. One possibility is sucrose density gradient separation, whereby IgM was successfully isolated from Fraction D. However, these results should be viewed with caution since Fraction D contained only IgG and IgM. The situation might be different if IgA was also present and there is evidence that colostrum of some cows does contain this immunoglobulin.

Neither the sucrose density gradient system nor Smith's procedure separated IgG1 from IgG2. Other isolation procedures are needed to separate them.

References