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Proteins of Human Milk. I. Identification of Major Components

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Traditionally, human milk proteins are identified largely by reference to bovine milk. Hence, to identify the major proteins in human milk, we subjected human and bovine milk, in parallel, to high-resolution two-dimensional electrophoresis. Isoelectric precipitation at pH 4.6 was our criterion for distinguishing whey proteins from those of the casein complex. The α- and β-caseins were identified on the basis of relative abundance, relative molecular mass, and relative isoelectric points. Kappa casein was identified as a series of four spots, which disappear from bovine skim milk treated with rennin (chymosin; EC 3.4.23.4) during the clotting process. Para κ-casein does not appear on the standard ISO-DALT pattern after treatment of bovine milk with rennin, but does appear in BASO-DALT patterns, indicating its high isoelectric point. No protein disappeared from ISO-DALT patterns of human milk after rennin treatment, and no new protein comparable to bovine para κ-casein appeared in the BASO-DALT patterns; this suggests that κ-casein is absent from human milk. The proteins identified in human milk patterns include the α and β casein families, lactalbumin, albumin, transferrin, IgA, and lactoferrin. Numerous additional proteins seen in patterns for human milk remain to be identified.

Additional Keyphrases: *ISO-DALT · BASO-DALT · caseins · whey proteins · inter-species differences · lactalbumin · lactoferrin · colostrum/milk differences · review of studies on milk proteins · two-dimensional electrophoresis · position shift with one-unit change in charge · mechanism of rennin action · post-translational modifications*

Until relatively recent times, human milk was essential to the survival of both the individual infant and of the human species. Despite its importance, it is rarely examined in the clinical laboratory, and many of the proteins it contains are not well characterized. In contrast, bovine (and to a lesser extent ovine) milk proteins have been extensively studied (reviewed in refs. *1–12*). Comparative studies suggest that there are only a few major classes of milk proteins (*7*), hence the nomenclature of bovine milk proteins has been carried over to those of man (reviewed in refs. *13–14*).

With the resurgence of interest in breast feeding, and with the development of high-resolution methods for protein analysis (*15–21*), it becomes of interest to re-examine human milk proteins by use of these new techniques, in order to compare human and bovine milk in detail, to note differences between colostrum and mature milk, and ultimately to explore changes that may be of genetic, nutritional, or disease origin. This paper presents both a review of previous studies on milk proteins that may be of interest to clinical chemists and a comparative analysis of bovine and human milk by use of high-resolution two-dimensional electrophoresis.

The major milk proteins are extensively modified post-translationally, with the result that the products of a single gene may appear in milk as a series of proteins that differ in charge or mass, or both, as has been observed to be the case for several human plasma proteins (*22*).

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Clinical Interest in Milk Proteins

Synthesis of milk proteins. The major ovine milk proteins are synthesized as larger precursors (*23*); are clipped by a microsomal protease; may be postsynthetically modified in the Golgi apparatus by the addition of phosphate, sugars, and sialic (neuraminic) acid; and are then excreted. The clipped segments of these proteins may be important signals within the mammary gland, and consist of amino-terminal fragments of 15, 21, 18, and 19 amino acid residues for the three calcium-sensitive caseins (α_{s1}, α_{s2}, and β), κ-casein, β-lactoglobulin, and α-lactalbumin, respectively. The details of milk-protein synthesis in man do not appear to have been explored at this level.

Milk secretion. The fat globules of milk bud off breast exocrine cells and are covered with authentic cell membrane, which may be isolated and analyzed (*3, 24–36*). Specific surface-differentiation antigens of human mammary epithelial cells have been isolated from membranes of fat globules of human milk (*28*). Casein micelles and soluble (whey) proteins are collected into secretory vesicles for exocytosis (*31*). Microtubules appear to be involved in milk secretion, because milk production is inhibited by colchicine and vinblastine (*26*). The composition of membranes that sediment with casein in bovine milk differs slightly from that of membranes isolated from fat globules of milk (*25, 26*).

Cell and cell fragments in milk. Milk contains various cell types, and cell fragments (*37–45*). The number of nucleated cells in human milk is about 1000-fold that in bovine milk (*40*). In the latter, polymorphonuclear leukocytes, macrophages, and monocytes are seen, plus anucleate cell fragments (*39*). In goat's milk, cell fragments containing abundant rough endoplasmic reticulum, lipid droplets, protein granules, and a few Golgi complexes have been reported (*45, 46*). The lymphocytes of bovine milk are about 45% T and 20% B cells (*40*). Because only a few viable cells are required for protein mapping by use of radiolabels, cells isolated from milk appear to be suitable samples for two-dimensional mapping.

Caseins. Caseins are phosphorylated proteins that are unique to milk, precipitate isoelectrically at pH 4.6, are almost exclusively present as micelles in normal milk, and normally bind nutritionally important calcium. All caseins are post-translationally modified. The major classes of bovine caseins, originally defined on the basis of electrophoretic mobility, include α, β, γ, and κ casein. Mature bovine milk contains (as a percentage of total skim-milk proteins) α_{s1}-casein, 39–46%; α_{s2}-casein, 8–11%; β-casein, 25–35%; κ-casein, 8–15%; and γ-casein, 3–7% (*11*). Caseins make up about 81% of the total protein in bovine milk, but only 30% of the proteins of human milk (*14*). Human milk proteins are mostly whey proteins.

α-Casein. α_{s1}-Casein is the major protein of bovine milk; the remainder of the α-casein (α_{s0}-, α_{s2}-, α_{s3}-, α_{s4}-, α_{s5}-caseins) is present in variable (but always small) amounts (*4*). The complete amino acid sequences of the major bovine variants are known (*4*). The most common genetic variant is $\alpha_{s1}\beta$, which has a chain of 199 residues, eight of which are phosphorylated serines (*7*). In contrast, human α-casein is not the major casein, and although it is known to resolve into three

bands electrophoretically (*47*), it is not well characterized (*13*).

β-Casein. Bovine β-caseins include several genetic variants having chains of 209 amino acids, not including (except for one variant) five phosphoserines (*7*). In man, β-casein is the major casein, and it is not uniformly phosphorylated, consisting of a single peptide chain having 0–5 phosphorylated residues, hence exhibiting six bands on electrophoretic analysis (*48*, *49*).

γ-Caseins. A minor and little-studied fraction of bovine casein (termed "γ-casein") consists largely of fragments of β-casein (*4*, *7*).

κ-Casein. In the calf stomach, bovine κ-casein is split by rennin (chymosin; EC 3.4.23.4) into paracasein, which precipitates casein micelles to form curds, and into a macropeptide (*50–52*) that is soluble in 120 g/L trichloroacetic acid (TCA). Bovine κ-casein is glycosylated, sialated, and phosphorylated, resulting in considerable electrophoretic heterogeneity (*53*). Electrophoretic analysis of human milk reveals a small amount of protein with the mobility of κ-casein, and fractions have been isolated from human milk that yield trichloroacetic acid-soluble peptides on treatment with rennin (*54*). Doubt persists, however, regarding the existence of true κ-casein in human milk (*13*, *14*), especially since the human stomach does not produce rennin (*12*).

α-Lactalbumin. A major whey protein, α-lactalbumin, is thus far found in all mammalian milks that contain lactose (*7*, *55*). UDP-galactose:*N*-acetylglucosamine galactosyltransferase normally synthesizes *N*-acetyllactosamine. However, in the presence of α-lactalbumin, which acts as a "specifier" protein (*56*), lactose is synthesized. The primary structure of α-lactalbumin is known (*4*, *57*).

β-Lactoglobulin. Bovine milk contains two β-lactoglobulins, each consisting of 162 amino acid residues. The two major variants (A and B) differ by one amino acid and by one charge (*4*). This protein is absent from human milk.

Immunoglobulins. Both human and bovine colostrums contain high concentrations of immunoglobulins initially, which decline rapidly as the nature of the milk being produced becomes more mature. Approximately 86% of bovine colostrum immunoglobulins are IgG, and ~2% IgA. In contrast, about 90% of human colostral immunoglobulins are IgA, about 2% IgG (*58–60*). The immunoglobulin concentration is very high in colostrum, but declines rapidly in the first few postnatal days as the milk matures (*58–60*).

Other milk proteins. Lactoferrin, an iron-binding protein, is found in both bovine (*11*, *61*) and human (*13*, *62–66*) milk. In its native state it is only partly saturated with iron; thus it can serve the double function of competing with bacteria for iron to produce a bacteriostatic effect and of providing iron when digested. Small amounts of albumin, transferrin, and other plasma proteins leak into both bovine and human milk. More than 30 enzymes have been described in milk (*67*), only a few of which are thought to serve some function after excretion. Among the more interesting of these are γ-glutamyltransferase (EC 2.3.2.2), which is important in amino acid transport (*35*, *36*, *68*, *69*), xanthine oxidase (EC 1.2.3.2) (*70–72*), lipases (*73*, *74*), superoxide dismutase (EC 1.15.1.1) (*75*, *76*), acid phosphatase (EC 3.1.3.2) (*77*), DNA polymerase (EC 2.7.7.7) (*78*), neuraminidase (EC 3.2.1.18) (*79*), lysozyme (EC 3.2.1.17) (*59*), and sialyltransferase (EC 2.4.99.1) (*80*). Both α_2-microglobulin (*81*), and β_2-microglobulin are present in human colostrum and milk (*82*). Certain proteins in milk specifically bind vitamin B_{12} (*83*), folate (*84*, *85*), corticosteroids (*86*, *87*), and zinc (*88–91*). Thermoprecipitable proteins have been described in both bovine (*92*) and human (*93*) milk.

Resistance to infection. Human milk possesses antibacterial, antiviral, and anti-allergic properties, which have been frequently reviewed (*13*, *38*, *41*, *60*, *94–106*). Resistance factors include specific immunogobulins (*94–105*); lysozyme (*59*, *67*); lactoperoxidase (EC 1.11.1.7) (*107*); the so-called bifidus factor in human milk, which stimulates the growth of *Bifidobacterium bifidum* in the infant intestine (*13*, *14*); *N*-acetylneuraminic acid; and proteins that bind iron, folate, and vitamin B_{12} (*108*). A component of the complement system, C3, is found in very high concentrations in colostrum, but rapidly declines as milk matures (*60*, *109*). The human infant's initial supply of protective immunoglobulins is obtained via the placenta (*110*); only a small amount is absorbed from colostrum (*111*). The reverse is true for calves, which absorb immunoglobulins into the blood stream from colostrum (*103*). In mice, maternal interferon is passed to the offspring via milk (*112*).

Drugs and contaminants in milk. At least seven categories of contaminants or residues can be found in milk (*14*): hormones, drugs, metals and their organic-compound derivatives, pesticides, herbicides, fungicides, and disinfectants.

Maternal nutrition. Most studies on compositional changes in milk as a function of maternal nutrition have centered on changes in lipids (*14*) or on gross milk composition. No systematic studies have been done on variations in each of the many known human milk proteins as a function of nutrition.

Milk proteins and cancer. The relation between human milk proteins and human cancer has been reviewed by Laurence (*113*), and breast tumors have been shown to produce an array of appropriate and inappropriate proteins, including hormones, immunoglobulins, oncofetal antigens, and milk proteins (*114*). A number of oncovirus-related proteins have been described in human milk, including a major core protein (*115*), a GP-55-like protein in human breast cancer (*116*), and a protein related to the major envelope protein of murine mammary tumor virus (*117*). Among the milk proteins studied in relation to cancer are a folate-binding protein (*118*), vitamin-B_{12} binding protein, and lactalbumin (*119*).

Rat and mouse milk proteins. Some of the major proteins of milk from mice (*120*, *121*) and rats (*122–124*) have been characterized.

Materials and Methods

Electrophoretic Analysis

Two-dimensional electrophoresis—isoelectric focusing in acrylamide in the presence of 8 mol/L urea and NP-40 detergent, followed by acrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS) in the second dimension—was done with the ISO-DALT system as previously described (*16*, *17*, *125*). All patterns were made visible by staining with Coomassie Brilliant Blue. Internal-charge and molecular-mass standards were used (*126–128*). Samples were prepared by mixing with 8 mol/L urea in volume ratios of 1:1, 1:2, and 1:3, and stored at −70 °C if not analyzed promptly.

Results

Identification of Major Bovine Milk Proteins

For basic and acidic proteins that would not focus under equilibrium conditions, nonequilibrium pH-gradient electrophoresis was used (*129*), as adapted either for basic proteins (*130*) (out to histones), or for very acidic proteins such as are found in urine (*131*).

Because human milk proteins are partly identified by reference to bovine milk, we first describe our results for bovine milk. Orienting studies were done with commercial pasteurized whole or skim milk; the definitive studies were performed

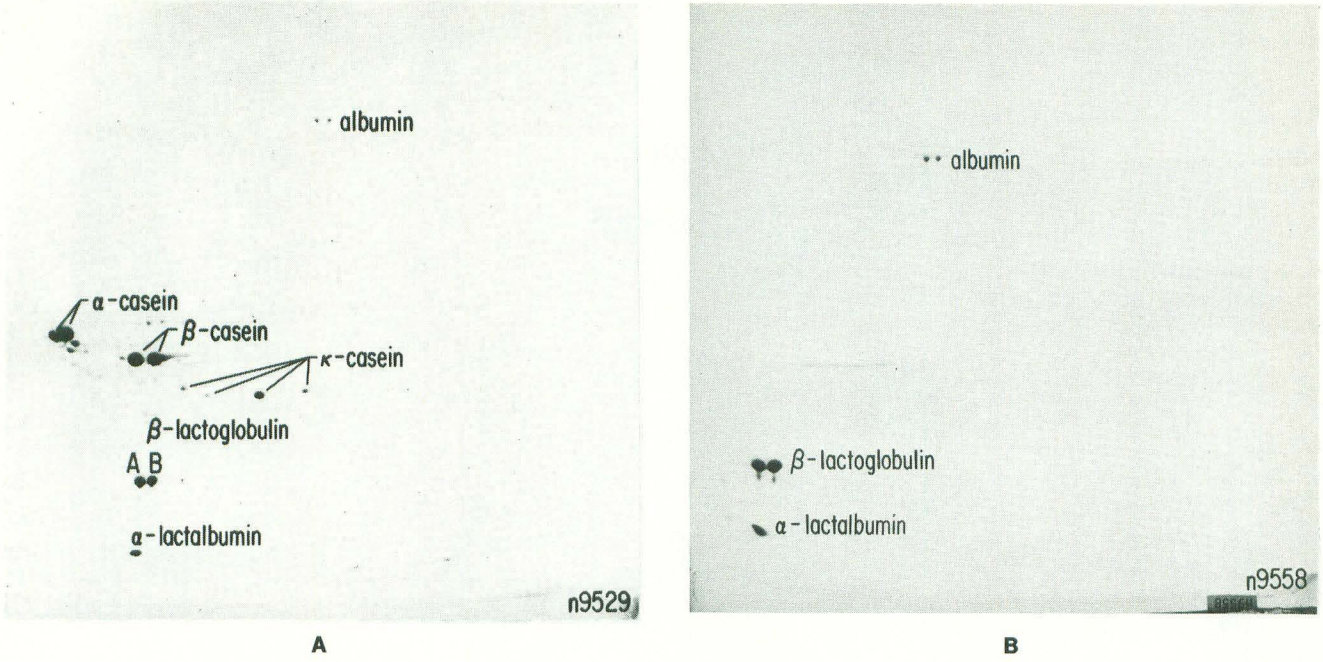

Fig. 1. Two-dimensional electrophoresis patterns of bovine milk

Acid end of isoelectric focusing gel (first dimension) is to the left. Methods for spot identification are described in text. *A*, pattern of whole milk. Lactoferrin has moved off the pattern to the right (basic) side. Isoelectric focusing is to equilibrium with LKB Ampholines, 90% pH 3.5–10 and 10% pH 5–7. *B*, supernatant whey after rennin clotting of whole bovine milk. Note complete absence of the entire casein complex. Electrophoresis as in Fig. 2

on one fresh sample of Guernsey milk. It was centrifuged (2000 × *g*, 15 min, 20 °C) and the cream supernatant and the cell and debris pellet were discarded. The skim milk was then stored at −70 °C until analyzed. Figure 1*A* shows patterns obtained with unfractionated bovine skin milk; the pattern of the whey remaining after isoelectric precipitation of casein is shown in Figure 1*B*. Identifications were done as follows.

α-Lactalbumin. The spot identified as α-lactalbumin is the only major spot with an SDS molecular mass in the expected position at about 14 000 daltons, was not isoelectrically precipitated at pH 4.6, remained in the whey after rennin treatment, and was essentially homogeneous in both dimensions in two-dimensional electrophoresis.

β-Lactoglobulin. Identification of the β-lactoglobulins was based on their mass (18 362 daltons for genetic variant A and 18 276 daltons for genetic variant B), and on their presence in the whey after isoelectric precipitation at pH 4.6 or rennin treatment. Genetic variant A has aspartic acid at position 64, for which is substituted glycine in variants B and C (*4*), resulting in a difference of one charge unit. Hence, A should focus at a slightly more acid position than B or C. This fact allows variants A and B to be identified in Figure 1. Note the slight heterogeneity exhibited by β-lactoglobulin in the SDS dimension.

Bovine plasma albumin. This was identified by position and by comparison with the position of authentic purified protein.

Lactoferrin. Lactoferrin was identified by its position in the SDS dimension corresponding to its mass of 78 000 daltons (*64*) and by co-electrophoresis with purified human lactoferrin. Additional confirmation by use of authentic bovine lactoferrin is needed.

α-Casein. The major protein of bovine milk is α_{s1}-casein (M_r 22 068). While its calculated relative molecular mass (*4*) is less than that of bovine β-casein, in SDS its apparent relative molecular mass is known to be greater (*132*), accounting for its position in Figure 1*A*. The protein spot identified as α-casein was in the curds produced by isoelectric precipitation or rennin treatment, and also was present in the casein micelle pellet produced by centrifugation of skim milk at 100 000 × *g* for 1 h. In addition, the iso-ionic points for α-caseins are more acid (*133*) than for β-caseins (*134*); as expected, α-casein focuses to the left of β-casein in Figure 1*A*.

β-Casein. Bovine β-casein was identified by its isoelectric focusing position (i.e., focusing at a less acid position than α-casein), by its molecular mass position (*ca.* 24 000 daltons), by its lower abundance than α-casein, by co-sedimentation with casein micelles during high-speed centrifugation, and by sedimentation with the curds after pH 4.6 isoelectric precipitation or treatment with rennin.

The primary translation products of the ovine mRNA for β-casein obtained with a wheat-germ cell-free system has an amino terminal extension of 15 amino acids (M_r = 1700), constituting a "signal" peptide, which is cleaved during intracellular processing (*23*). In Figure 2, two sets of two spots are seen above and slightly to the right of the major β-casein spots. If the bovine precasein resembles the ovine molecule, and if the signal sequence contains one basic and no acid residues and has a similar relative molecular mass, then accidentally secreted (leaked) pre-β-casein would fall in the position of one of these spot pairs. Further study will be required to determine whether these spots are indeed β-casein pre-proteins. The position is approximately correct for the mass increase involved (SDS dimension), and is to the right about one charge unit, as would be expected from the addition of one basic residue. Note that the distance one charge unit shifts a spot position differs for different proteins.

κ-Casein. Identification of κ-casein, which is heterogeneous both with respect to mass and charge, is based in these studies on appearance in the expected position with respect to molecular-mass range, on cosedimentation with casein micelles during high-speed centrifugation, and on precipitation at pH 4.6. Final identification, however, is based on its sensitivity to rennin. It is known that κ-casein is post-translationally modified and that these modifications, which produce both charge and mass heterogeneity, are all located in the smaller of the two pieces produced by rennin cleavage—the so-called macropeptide (*53*). The larger fragment, the para κ-casein,

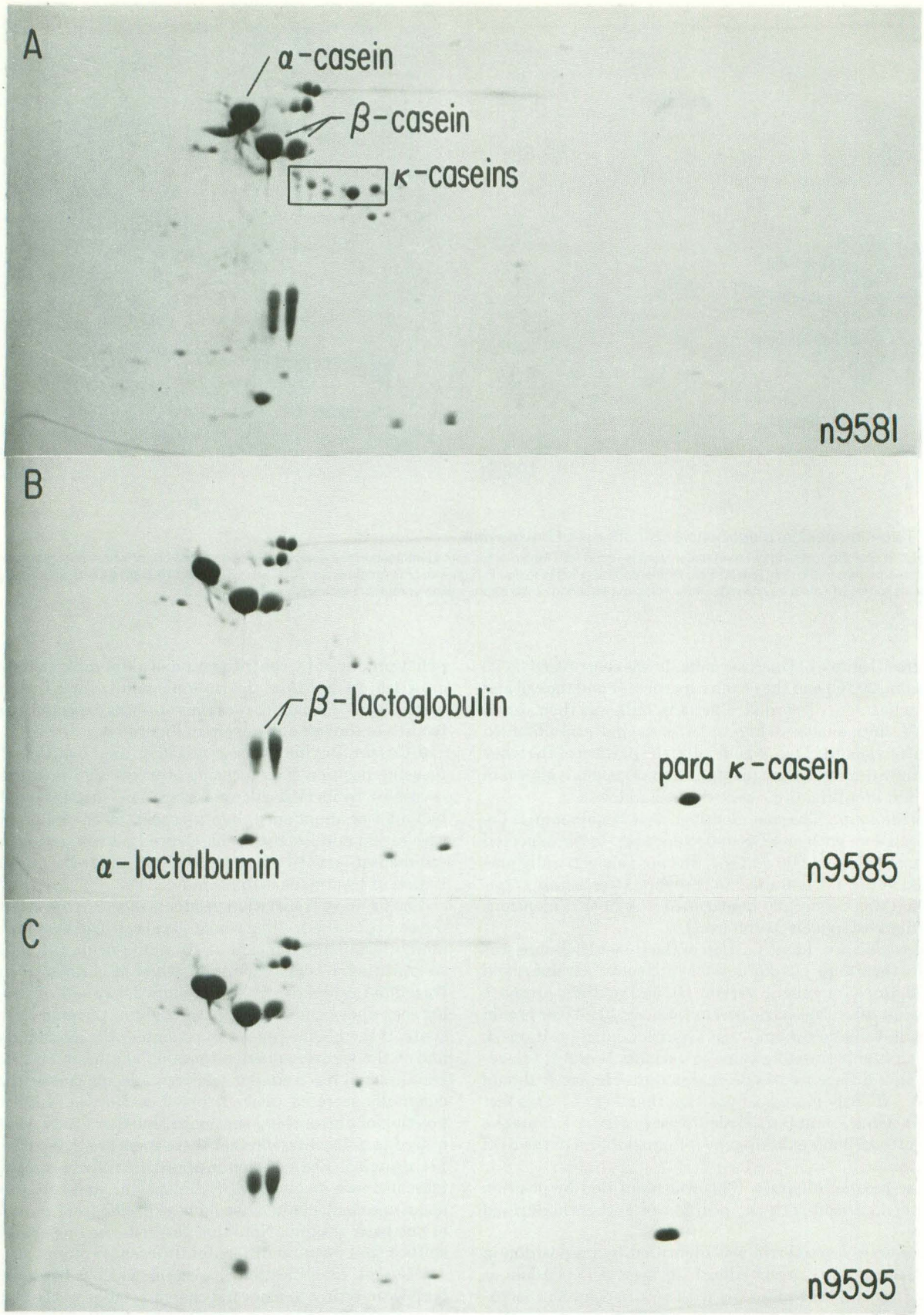

Fig. 2. BASO-DALT (non-equilibrium pH-gradient electrophoresis) of Holstein skim milk before and after treatment with rennin

10-μL samples of milk mixed with two parts 8 mol/L urea with NP40. *A*, before adding rennin, *B*, 4 min after adding rennin in concentration of 0.2 mg rennin (Sigma, 20 units activity per mg) to 10 mL of milk at 30 °C. Clotting was not yet evident. Only traces of κ-casein remain. Note appearance of para κ-casein. *C*, pattern observed after 20-min incubation with rennin

is homogeneous with respect to both charge and mass. Inspection of the amino acid sequence of κ-casein reveals that the macropeptide has an excess of acidic groups (phosphate, sialic acid, acidic amino acids), while para κ-casein has an excess of basic amino acids, and hence should focus at a very high pH—in fact, off of conventional equilibrium gels such as shown in Figure 1 in a right-hand direction. Using the BASO-system (*130*) of nonequilibrium pH gradient electrophoresis, we devised conditions so that the casein complex of spots and paracasein would all appear on the same pattern. We then added purified rennin to skim milk at 30 °C, and removed aliquots at intervals, mixed them with 8 mol/L urea, and stored them at −70 °C until analysis. Figure 2 shows the analysis of a series of aliquots from such an experiment. The milk was observed to clot in 5 min, about the interval required for all the spots identified as κ-casein to disappear and for the paracasein to appear. Previous studies have shown that when milk is clotted by action of rennin, nearly all of the κ-casein has been split (*51*), as measured by the rate of appearance of the acid-soluble macropeptide; this is in agreement with the results reported here. Rennin, examined electrophoretically, did not overlap with any of the milk proteins. The rennin was electrophoretically heterogeneous, as previously reported (*135*). In the experiments shown, the concentration of rennin was too low to be detected in the patterns.

The casein precipitated by rennin was washed and analyzed as shown in Figure 3*A*. It contained both α- and β-casein and the spot identified as para κ-casein, but lacked α-lactalbumin and β-lactoglobulin. Both of the latter were in the whey (supernate), as shown in Figure 3*B*.

Minor proteins. Numerous minor spots appear in patterns of heavily loaded samples that are not identified or described by coordinate position here. These will be described in studies recorded elsewhere, as will studies on membrane proteins of milk fat-globules.

Comparative Analysis of Human Milk

Human milk samples were obtained from five donors, beginning immediately after delivery and continuing on until the milk assumed its mature character. A representative pattern for human colostrum is shown in Figure 4; more mature milk is shown in Figure 5. Identifications were done as follows:

α-Lactalbumin: α-Lactalbumin was identified by reference to bovine milk, by comparison with a pattern obtained for an authentic human α-lactalbumin sample, and by reference to SDS–molecular mass standards. In addition, the spot appeared undiminished in all whey preparations examined (Figure 6).

The human casein complex. On one-dimensional electrophoresis, three bands are observed for α-casein and six for

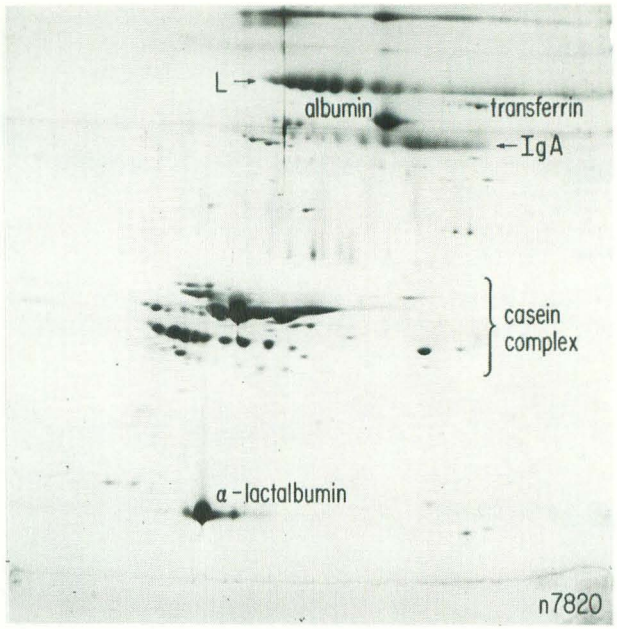

Fig. 4. ISO-DALT pattern of human colostrum obtained on first day after parturition

Note the complexity of the casein complex, and the lack of β-lactoglobulin. Row of nine spots above and to the left of albumin marked *L* may include UDP-galactosyltransferase. IgA is very heterogeneous in charge, giving the long streak immediately below albumin

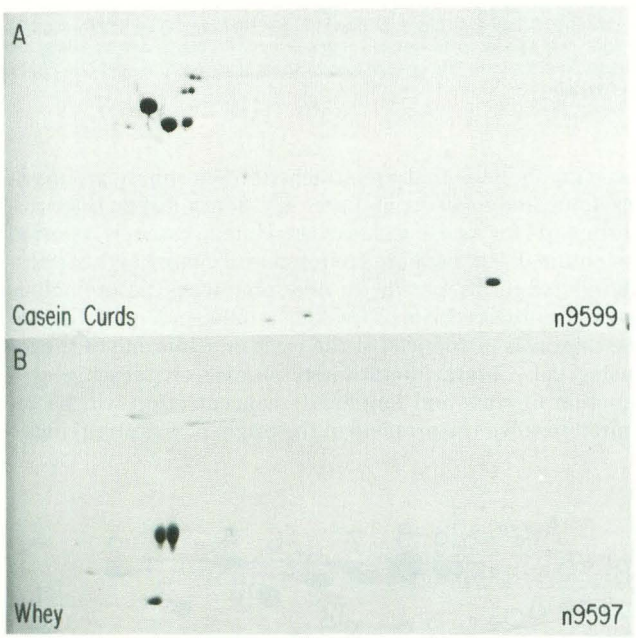

Fig. 3. *A*, Lower portion of BASO-DALT pattern of washed clotted casein from rennin experiment illustrated in Figure 2

Note para κ-casein is included in the precipitated protein, and α-lactoalbumin and β-lactoglobulin are absent

B, Whey from BASO-DALT experiment in Figure 2

Note absence of both α- and β-caseins *and* para κ-casein

Fig. 5. ISO-DALT pattern of human milk collected the fifth day after parturition

Note the decrease in IgA

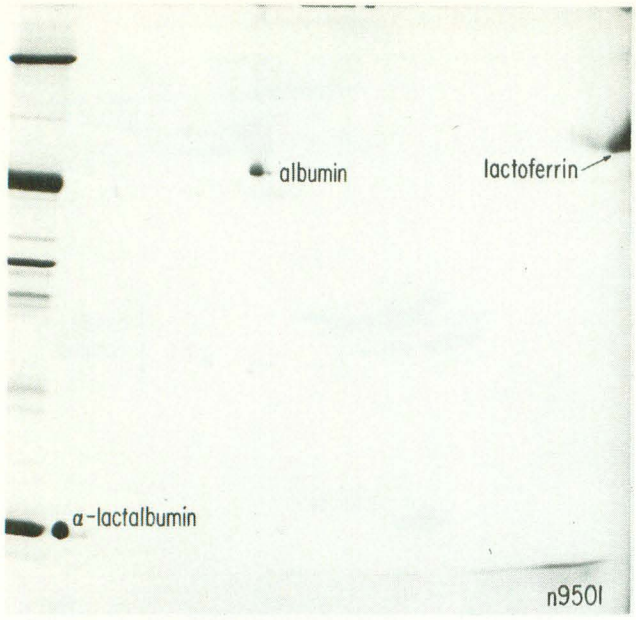

Fig. 6. ISO-DALT pattern of human milk whey obtained by isoelectric precipitation of caseins at pH 4.6
Note the complete absence of casein complex

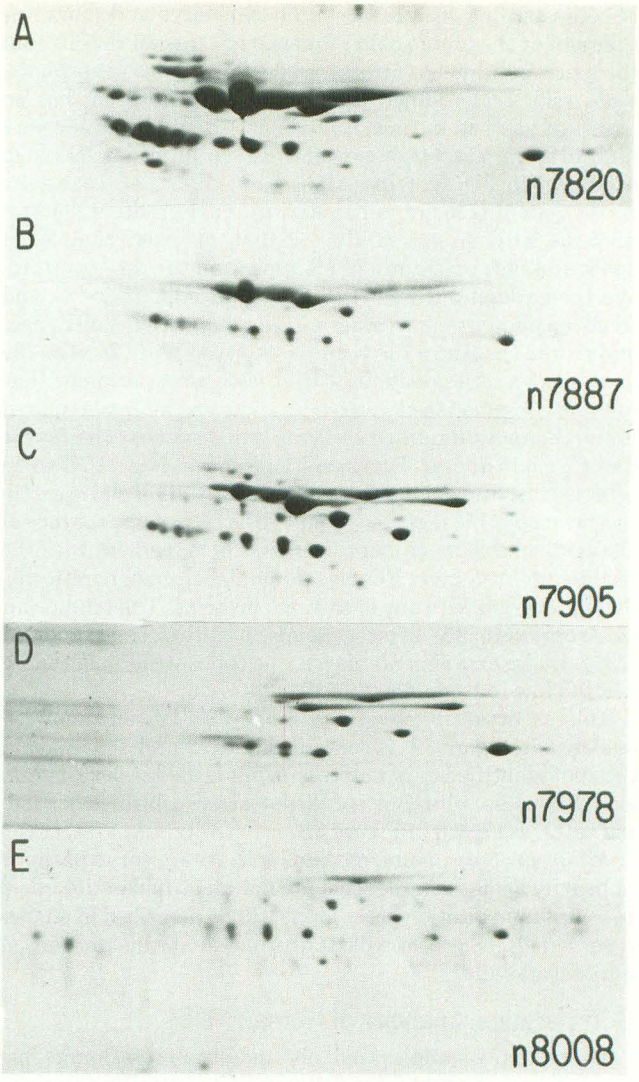

Fig. 7. ISO-DALT pattern of casein complex in milk from a single donor obtained at various intervals after birth
A, day one; *B*, day 3; *C*, day 4; *D*, day 6; *E*, day 10. Note the variability in ratios of spots that appear to be variants of one protein. Problems with streaking are discussed in the text. The gradual loss of spots in zones *A*, *B*, and *C* in Figure 8 is evident

β-casein. Note that these have been defined by electrophoretic position and by solubility characteristics, but it remains to be shown conclusively which bands are post-translationally modified versions of products of single genes. The patterns obtained by high-resolution two-dimensional electrophoresis show an unexpected complexity, and no assignments can be made with certainty without much additional study. The first problem is to find out which of all the many spots seen in the casein region are classical caseins, in the sense of both being precipitated isoelectrically at pH 4.6 and sedimentable in native milk under conditions in which casein micelles sediment. The pattern of isoelectrically precipitated casein includes nearly all of the spots seen in the casein region of patterns of unfractionated milk. The problems associated with finding out which proteins are post-translational modification variants of the same protein-gene-product are many, and are illustrated by Figure 7, which shows the human casein complex in milk obtained from a single donor at different times postpartum. Figure 8 shows the complex schematically.

From comparison of Figures 7 and 8 it is at once apparent that human casein has many more components than was previously thought. The spots shown in the delineated area A, B, and C in Figure 8 appear in colostrum, then disappear as the milk being produced becomes more "mature" in character. Spots 10–14 are tentatively identified as α-casein, which has not been previously resolved. The pair of spots 11 and 12 are useful orientation landmarks. This spot series could all be derived by post-translational modification from spot 10. An increase of one negative charge (or loss of one positive one) could shift the protein from position 10 to 11, while the addition of carbohydrate or neutral amino acids could increase the mass from position 11 to 12; additional charge changes could result in spots 13 and 14. Stair-step runs of spots sloping upward and to the left are frequently seen in plasma—for example, in the α_2HS glycoprotein—and appear to be characteristic of proteins marked for export from cells. The filled-in spots, 1–9, are tentatively identified as a series of post-translational modifications of β-casein. Spots 2 and 4 are quite variable and may be due to noncovalently attached groups that are slowly lost during isoelectric focusing, resulting in the "melting" of spot 2 into 6, for example, and 4 into 7. The attached groups may be present in milk initially, or may be basic ampholytes that are attached to phosphate groups of casein during focusing and are only slowly lost as the equilibrium pH for focusing is reached. Human casein is reported to contain 0–5 phosphate groups, and the more highly phosphorylated forms may be the ones producing the anomalous streaking observed. Such streaking is independent of whether the sample is introduced at the basic or acidic end of the focusing gel. Careful quantitative studies on focusing as a function of time and ampholyte concentration will be required to solve the problem of the origin of this streaking.

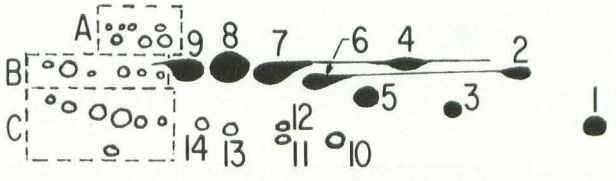

Fig. 8. Diagrammatic presentation of spots present in the human casein complex
Spots in zones *A*, *B*, and *C* disappear as milk matures. Spots *1–9* are tentatively identified as post-translational modifications of β-casein, which is the major casein of human milk; spots *10–14* are identified as α-caseins

If, as previous studies suggest, human β-casein contains 0–5 phosphate groups, then six spots should be seen in a horizontal row. Disregarding spots 2 and 4, we end up with one spot too many, i.e., seven (counting spots 1, 3, 5, 6, 7, 8, 9). The amount of protein in each spot varies as the milk matures, as has been reported for β-caseins. All of the proteins represented by spots 1–9 precipitate at pH 4.6, which suggests that all are caseins. With antibodies specific for β-caseins it should be possible to determine whether an additional and unexpected charge variant of β-casein exists, or whether one spot is due to a hitherto-unreported casein. This is part of the general problem of identifying all of the post-translational modifications of the product of one gene.

The human κ-casein problem. Rennin is a very weak protease—i.e., it will attack caseins other than κ-caseins, given sufficient time. Because human milk does not clot with rennin (*12*, *14*) and the human stomach does not make rennin, a human analog of such κ-casein may not exist. Reportedly, κ-caseins are present in human milk and yield acid-soluble peptide material after exposure to rennin. This result may have been due to nonspecific protease activity by rennin or to proteolytic contaminants in the rennin. A true human analog of bovine κ-casein would be heterogeneous with respect to charge and mass, be split at approximately the same rate by rennin as bovine κ-casein, and simultaneously would give rise to a homogeneous basic paracasein. Figure 9 demonstrates that the proteins of human κ-caseins are unchanged by treatment with rennin.

Lactoferrin. The position of lactoferrin was determined by comparison with a purified sample generously provided by Dr. A. Bezkorovainy of Rush Medical School (Figure 6).

Plasma proteins. The positions of albumin and transferrin were identified by reference to previously published maps (*22*), and to standards for molecular mass (*128*) and charge (*126*, *127*).

Comparison of Colostrum and Mature Milk

Human colostrum contains relatively large amounts of IgA (Figure 4). By comparison with IgA previously identified in human plasma (*136*), the position of IgA in colostrum (Figure 5) was determined, and the position partly confirmed by its rapid decrease in amount as the milk matured. Additional confirmatory studies are required to determine whether proteins other than IgA are present but obscured in the IgA region.

The major change observed as colostrum changes into mature milk is in the IgA, and in the loss of the families of spots in the casein complex shown as *A*–*C* in Figure 8.

Discussion

The major proteins in high-resolution two-dimensional maps of bovine milk have been identified, including α-lactalbumin, the two β_2-lactoglobulins, α- and β-casein, four post-translationally modified κ-chains, para κ-casein, lactoferrin, and albumin. Small spots were seen on either side of most major proteins, which could be charge variants produced either by incomplete post-translational modification (including sialylation or phosphorylation). In addition, on slightly overloaded gels, spots were seen that may be preproteins that were not cleaved during the intracellular processing. In some milks, especially goat milk, many cell fragments are present, which contain Golgi fragments and pieces of endoplasmic reticulum. These may also contain incompletely processed pre-caseins. Enough of these putative preproteins are seen to suggest that it is worth the effort to isolate them in sufficient quantity to identify them definitively, and to use them in experimental studies on intracellular protein processing.

The components of the casein complex have been identified by mapping the fraction isoelectrically precipitated from bovine skim milk at pH 4.6, the casein micellar fractions that are sedimented at high speed, and the clotted fraction after treatment with calf rennin. The major α-casein of bovine milk, while having a lower relative molecular mass than β-casein, as judged from amino acid sequence data, has a higher SDS-molecular mass, in keeping with previous observations (*132*). The position of the κ-caseins was determined by difference, i.e., by observing which protein spots disappeared during the time required for rennin to clot bovine milk. Rennin appears to have a weak proteolytic effect on other milk proteins, as judged from the slow proteolysis observed in cheeses made with use of rennin.

We suggest here both a revised definition of κ-casein, and a new mechanism for rennin clotting of milk. We propose that true κ-caseins have the following properties. Sequence studies on bovine κ-casein show it to be a single peptide chain with multiple post-translational modifications limited to the 64 N-terminal amino acids. These modifications are not uniform, and result in a series of variants, differing chiefly in charge and to a lesser degree in mass. The intact molecule, like the other caseins, has an acid isoelectric point, binds calcium, and attaches to casein micelles in a manner that allows it to be readily accessible to rennin. The action of rennin, which is

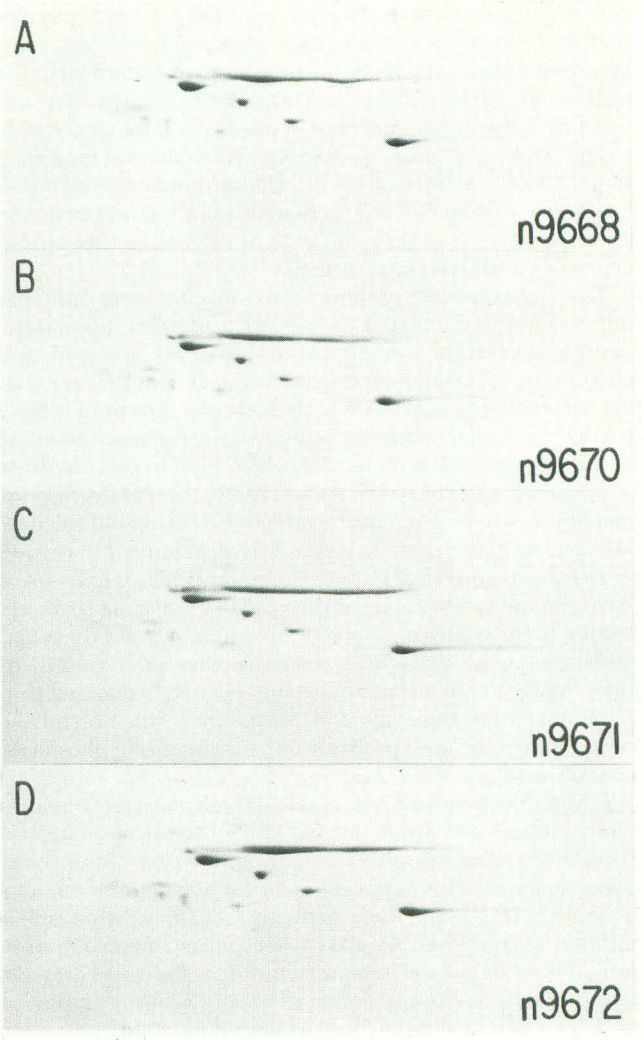

Fig. 9. ISO-DALT pattern of casein complex of human skim milk (47th day after parturition)

A, Before rennin additions as described in Figure 4. *B*, 3 min after rennin addition. *C*, 6 min after rennin addition. *D*, 20 min after rennin addition. In this experiment and in others with use of BASO-DALT technique no disappearance of spots from the casein complex has been seen, and no para κ-casein spot appeared in the basic (right-hand side) portion of the BASO-DALT patterns

essentially complete in the time required for rennin to clot milk, is to cleave the κ-casein chain into a macropeptide having an excess of acidic amino acids over basic ones, and all of the sialic acid, carbohydrate, and phosphate groups. The remaining large fragment, para κ-casein, is homogeneous in charge and mass, possesses no post-translational modifications, and has a large excess of basic amino acid residues as compared with acidic ones, giving it an alkaline isoelectric point. We propose that only proteins with all of these properties be considered true caseins.

The mechanism we propose for rennin clotting of milk is as follows. The section that is split off to become the macropeptide is external, contributes to the overall acidic pI, and also creates an asymmetric distribution of charges such that the molecule can aggregate with itself and also bind loosely to casein micelles, as is suggested by electron microscopy of density-tagged lectin molecules, which bind to carbohydrate on κ-casein selectively (*29*, *137*). The entire casein-micelle, including κ-casein complex together with the bound calcium, is sufficiently hydrophilic and negatively charged to remain in suspension at the pH of normal milk. When rennin splits κ-casein, the two sections of the κ-casein molecule that contribute to the charge asymmetry are separated, and the major moiety left, para κ-casein, is positively charged. Experimentally, we have found that almost any positively charged protein—including lysozyme and protamine—will precipitate casein from milk, most probably by combining with phosphate or sialic acid groups, or both.

Given this definition of κ-casein, largely derived from previous studies and from the two-dimensional mapping recording here, we examined human milk first to identify as many major proteins as possible, to see if human milk contains a κ-casein fraction as here defined, and to see what major differences could be observed between colostrum and mature milk. No evidence for human κ-casein, as defined here, was found under conditions that split all the κ-casein in bovine milk, or when incubation was prolonged fourfold.

By comparison with bovine milk patterns, published data on molecular mass, and in some instances, analysis of purified samples, we identified human α-lactalbumin, lactoferrin, albumin, and transferrin. The casein complex was identified by analyzing isoelectrically precipitated casein, and casein micelles sedimented centrifugally. The complex is much more heterogeneous and complex than previously thought. For example, the β caseins could not be unambiguously resolved into fractions differing in degree of phosphorylation from 0–5 phosphate groups as previously described (*48*, *49*).

Spots derived from a single gene and made heterogeneous by post-translational modification can be definitively identified by several techniques. These include the search for genetic polymorphisms, which will move all related spots in one sample, by enzymatically removing the post-synthetically added groups (i.e., by treatment with neuraminidase, phosphatase, or both) and comparing patterns before and after such digestion, by cutting spots out of two-dimensional patterns and comparing the products of limited proteolysis by using the technique of Cleveland et al. (*138*), or by immunoprecipitating and mapping all of the modified proteins that react with a monospecific antibody. All of these approaches should be used. These techniques will, for example, allow the question of the relationship of the nine spots seen in the β-casein region to the six spots expected to be examined.

Milk offers unique opportunities for analytical study in clinical chemistry in addition to these mentioned. A thorough study of many samples should yield a number of new genetic polymorphisms, some of which may be disease-related. In addition, the many proteins available in the milk fat-globule membrane offer for study a unique prefractionated sample of cell membrane, which should be examined for cancer-related markers. Lastly, caseins may be easily removed from milk, leaving whey, which has only a few major proteins. These may be immunosubtracted (*139*), leaving an interesting collection of trace proteins, which may be either radiolabeled by methods previously described (*140*), or detected by silver staining (*141*, *142*).

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