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# Modulation of Secreted Proteins of Mouse Mammary Epithelial Cells by the Collagenous Substrata

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**ABSTRACT** It has been shown previously that cultures of mouse mammary epithelial cells retain their characteristic morphology and their ability to produce  $\gamma$ -casein, a member of the casein gene family, only if they are maintained on floating collagen gels (Emerman, J. T., and D. R. Pitelka, 1977, *In Vitro*, 13:316–328). In this paper we show: (a) Cells on floating collagen gels secrete not only  $\gamma$ -casein but also  $\alpha_1$ -,  $\alpha_2$ -, and  $\beta$ -caseins. These are not secreted by cells on plastic and are secreted to only a very limited extent by cells on attached collagen gels. (b) The floating collagen gel regulates at the level of synthesis and/or stabilization of the caseins rather than at the level of secretion alone. Contraction of the floating gel is important in that cells cultured on floating glutaraldehyde cross-linked gels do not secrete any of the caseins. (c) The secretion of an 80,000-mol-wt protein, most probably transferrin, and a 67,000-mol-wt protein, probably butyrophilin, a major protein of the milk fat globule membrane, are partially modulated by substrata. However, in contrast to the caseins, these are always detectable in media from cells cultured on plastic and attached gels. (d) Whey acidic protein, a major whey protein, is actively secreted by freshly isolated cells but is secreted in extremely limited quantities in cultured cells regardless of the nature of the substratum used.  $\alpha$ -Lactalbumin secretion is also decreased significantly in cultured cells. (e) A previously unreported set of proteins, which may be minor milk proteins, are prominently secreted by the mammary cells on all substrata tested. We conclude that while the substratum profoundly influences the secretion of the caseins, it does not regulate the expression of every milk-specific protein in the same way. The mechanistic implications of these findings are discussed.

The concept that the final expression of some genetic information may be regulated by extracellular matrix (ECM)<sup>1</sup> components has now gained support from several sources (for recent reviews, see references 1, 2) and has prompted consideration of the possibility that tissue-specific differentiation and maintenance of a differentiated phenotype may be influenced by the substratum to which the cells are attached. The mammary gland provides a striking example of such influences. Emerman and co-workers (3, 4) demonstrated that mammary epithelial morphology and the ability to produce and secrete  $\gamma$ -casein were retained by cultures of mouse mammary epithelial cells maintained on collagen gels that had been made to float in the culture medium. The same cells maintained on plastic culture dishes lost these differentiated

characteristics even in the presence of lactogenic hormones. Later studies of glucose metabolism and lactose synthesis, which do reflect the state of differentiation of the mammary cells, revealed that expression of these characteristics was modulated by the culture substratum (5, 6). Most recently, Wicha et al. (7) have shown that cells cultured on "biomatrices" prepared from rat mammary gland produce higher levels of  $\alpha$ -lactalbumin than cells cultured on other substrata.

The mammary epithelial cells in culture, therefore, provide a highly manipulatable system to study the mechanism by which the substratum influences the final differentiated phenotype. The system is particularly useful as milk proteins provide a ready access to the *in vivo* phenotype. It is therefore important to examine the entire pattern of secreted proteins in culture in order to assess the following: (a) whether the substratum can modulate the entire differentiation program of epithelial cells and influence production and secretion of

<sup>1</sup> Abbreviations used in this paper: ECM, extracellular matrix; WAP, whey acidic protein.

all the milk proteins, or whether it modulates expression of just one or two specific proteins; and (b) whether it is able to confer exclusiveness to the expression of differentiated products and to prevent the expression of proteins that are unrelated to milk proteins.

We approached these questions by analyzing milk proteins by two-dimensional gel electrophoresis and comparing these patterns with the pattern of proteins secreted into the culture medium by cells maintained on three kinds of substratum: tissue culture plastic, attached collagen gels, and floating collagen gels. We also investigated aspects of the mechanism by which the substratum can bring about its effect. We demonstrate that modulation does not occur at the level of secretion but occurs at a biosynthetic or degradative level. Furthermore we demonstrate that modification of the collagen gel by glutaraldehyde cross-linking leads to inhibition of its ability to permit casein secretion upon floatation. This observation is discussed in terms of possible mechanisms involved in substratum-cell interactions. An abstract of this work has appeared (8).

## MATERIALS AND METHODS

**Materials:** Medium 199, gentamicin, and fetal bovine serum were obtained from Gibco Laboratories (Grand Island, NY). Insulin (bovine pancreas) and kallikrein was obtained from Calbiochem-Behring Corp. (La Jolla, CA). Cortisol and prolactin (sheep) were purchased from Sigma Chemical Co. (St. Louis, MO), and Collagenase (Type II) from Worthington Biochemical Corp. (Freehold, NJ). [<sup>35</sup>S]Methionine (1,440 Ci/mmol) was obtained from Amersham (Arlington Heights, IL), [<sup>3</sup>H]tyrosine (52.9 Ci/mmol) from New England Nuclear (Boston, MA), SDS from British Drug Houses (Poole, U.K.), acrylamide from Bio-Rad Laboratories (Richmond, CA) or from Eastman Kodak (Rochester, NY). The latter was purified by charcoal adsorption before use. General chemicals were A.R. quality and were obtained from Mallinckrodt Inc. (St. Louis, MO) or Baker Co., Inc. (Sanford, ME). *Staphylococcus aureus* V8 protease was obtained from Miles Laboratories (Kankakee, IL). Anti-mouse albumin and transferrin antibody were obtained from Cappel Laboratories (West Chester, PA). Nitrocellulose membrane was obtained from Schleicher & Schuell (Keene, NH).

**Collection and Fractionation of Milk:** Milk was collected from lactating BALB/c mice (Cancer Research Laboratory, University of California, Berkeley). Mice were injected with oxytocin (2.5 IU) and milked 20 min later with a suction device. The milk was diluted with Tris-saline solution (0.125 M NaCl, 25 mM Tris HCl, pH 7.4, 5 mM KCl) containing phenylmethylsulfonyl fluoride (PMSF, 2 mM). Samples were centrifuged at 1,200 rpm (IEC HN-S centrifuge) for 10 min, the fat layer was carefully removed, and the skim milk was decanted from the cell pellet. The skim milk was then clarified by centrifugation one more time and the fat layer washed again in Tris-saline. Caseins were isolated from the skim milk by acid precipitation (pH 4.6). The precipitate was washed in acetone before solubilization for electrophoresis. To obtain radiolabeled milk proteins, we intraperitoneally injected three 6-d-lactating mice with a total of 2 mCi of [<sup>35</sup>S]methionine. 12 h later, milk was collected from the mice and fractionated as described.

**Epithelial Cell Isolation:** This was carried out according to the procedure of Emerman and Pitelka (3), except that a different dissociation medium was used to obtain better cell yield. Our dissociation medium contained collagenase (Type II, 0.3%), trypsin (0.15%), fetal calf serum (5%), F10 medium (0.98%), sodium bicarbonate (0.12%), and HEPES (0.26%). The hormones insulin, cortisol, and prolactin were added (5 µg/ml) just before use and were present throughout culturing unless otherwise indicated. Briefly, finely chopped tissue was dissociated at 37°C for 50 min. Undissociated tissue was then removed and redigested with fresh dissociation solution for 30 min. The two cell isolates were pooled, and epithelial cells were isolated by differential centrifugation. Cells were counted in a hemocytometer and plated at  $3 \times 10^5$  epithelial cells/cm<sup>2</sup> on either 35-mm plastic tissue culture dishes or 35-mm collagen gel-coated dishes in medium 199 containing 50 µg/ml gentamicin, 11 mM glucose, 10% fetal calf serum, and hormones at 37°C in 95% air and 5% CO<sub>2</sub>. Serum was eliminated from the medium after the first day in culture. Some of the collagen gels were made to float on the fourth day by one's going around the edge with a sterile spatula and gently shaking the dish (see below also). The medium was changed every other day.

In experiments in which Percoll gradients were used to check the purity of the cells, discontinuous gradients of Percoll (16 ml; 20% to 80%, in 10% steps)

in medium 199 were made. Cells ( $3 \times 10^6$ ) were loaded onto each gradient and then centrifuged at 2,350 rpm for 30 min (IEC Centrifuge, Needham Heights, MA; CFU 500 equipped with a 259 rotor). The cells distributed themselves at the three top bands. These were removed, the suspensions were diluted with medium 199, and the cells collected by centrifugation at 800 rpm for 3 min.

**Fibroblast Cell Isolation:** Mammary fibroblasts were isolated from the same dissociated tissue that was the source of epithelial cells. The suspension of dissociated cells was centrifuged at 800 rpm for 3 min (IEC HN-S centrifuge), and the epithelial cells were pelleted. The supernatant was taken and recentrifuged at 1,500 rpm for 5 min. The second pellet was washed to remove any remaining digestion enzymes and then the cells were plated in medium 199 with 10% fetal calf serum. This yielded a mixed culture containing fibroblasts and epithelial cells. Brief trypsinization of the mixed culture yielded cells that were plated in medium 199 with 10% fetal calf serum. After daily changes of medium, a confluent fibroblast culture was obtained.

**Preparation of Attached and Floating Collagen Gels:** This was carried out as described (9) using collagen from rat tails. Briefly, rat tail tendons were sterilized in alcohol and then extracted with 1:1,000 diluted acetic acid at 4°C for 48 h. Undissolved fibers were removed by centrifugation in sterile tubes at 13,000 g for 30 min at 4°C. Collagen concentration of the clear supernatant was ~2–3 mg/ml. To prepare collagen gels in 35-mm dishes, we mixed 0.88 ml of the stock collagen solution with 0.11 ml of 10 times-concentrated medium 199 and 0.11 ml of 0.34 N NaOH. The dish was placed in incubator until the gel had set. Before use, gels were equilibrated overnight with culture medium. When gels were cross-linked with glutaraldehyde, they were washed at 37°C on a gyrator shaker with six changes of phosphate-buffered saline to remove medium components, and then treated with 2% glutaraldehyde in phosphate-buffered saline at 37°C for 12 h. The glutaraldehyde was then removed and the gels were treated with phosphate-buffered saline containing 0.1 M glycine at 37°C for 30 min. This was repeated at least six times and the gels were finally rinsed three times with culture medium for 10 min.

**Radiolabeling Procedures:** Monolayer cultures were labeled 1 wk after plating using [<sup>35</sup>S]methionine (100 µCi/plate) in medium 199 (2 ml) with complete hormone complement and 11 mM glucose. Labeling was carried out for 24 h, unless otherwise indicated, after which time the medium was removed and the gels with attached cells were transferred to a microfuge tube and centrifuged (Beckman microfuge; Beckman Instruments, Inc., Palo Alto, CA) for 5 min. Medium trapped within the gel was released by this procedure and was pooled with the rest of the medium. Samples were stored at -70°C until analyzed.

Suspension cultures ( $6 \times 10^6$  freshly isolated cells) were labeled for 2 h at 37°C in methionine-free medium 199 (1 ml) with 11 mM glucose and complete hormone supplement. 100 µCi of [<sup>35</sup>S]methionine was included in the incubation mixture, and the suspension was gently shaken (90 rpm) on a gyrator shaker under an atmosphere of air/CO<sub>2</sub> (95:5).

**DNA Determination:** DNA determination was done according to Hinegardner (10). Recrystallized diamino benzoic acid was used in this fluorometric assay.

**Two-dimensional Gel Electrophoresis:** Proteins in skim milk and the culture medium were precipitated with trichloroacetic acid (10%) using *Micrococcus* cell wall as carrier. The precipitate was washed with cold acetone and dissolved in lysis buffer containing 9.5 M urea, 2.0% Nonidet P-40 (NP-40), 5% β-mercaptoethanol, 1.6% ampholine, pH 5–7, 0.4% ampholine, pH 3.5–10 (11). Equal counts were loaded for direct comparison. Nonequilibrium pH gradient electrophoresis (see reference 11) was carried out with gels containing 9.2 M urea, 4% acrylamide, 2% NP-40, 2% ampholine, pH 3.5–10, 0.2% ampholine, pH 5–7. Gels were electrophoresed at 400 V for 5 h. The second dimension was a SDS 6–15% acrylamide gradient gel employing a discontinuous buffer system (12) and was run at 5 W/gel. After fluorographs were taken, individual spots on the gels were excised, swelled, and solubilized in a cocktail containing nine parts NCS (Amersham) and one part 10% SDS (BDH) for 10 h at 62°C, followed by shaking at 37°C for 48 h. After addition of aquasol (New England Nuclear), the samples were counted by liquid scintillation spectrometry.

**Partial Proteolytic Digestion:** Peptide mapping by limited proteolysis in SDS was done according to Cleveland et al. (13). Digestion with *Staphylococcus aureus* V8 protease was carried out without prior elution of proteins from the gels.

**Immunoprecipitation:** Immunoprecipitation was carried out essentially as described by Lee et al. (14). Aliquots of skim milk, culture media, or cell extracts prepared in lysis buffer containing 25 mM Tris-HCl, pH 7.4, 50 mM NaCl, 0.2% NP-40, 0.5% deoxycholate, 2 mM phenylmethylsulfonyl fluoride and 200 U/ml of kallikrein were incubated with antibody at 4°C for 45 min, then absorbed with protein A-sepharose beads and subsequently washed three times with lysis buffer. When mouse monoclonal anti-rat α-casein antibody (which cross-reacts with mouse α-casein, see reference 14a) was used, the

incubation was prolonged to overnight and second antibody, goat anti-mouse IgG (heavy and light chain specific), was added before absorbing with protein A-sepharose beads. The immunoprecipitated proteins were dissociated from protein A-sepharose by boiling in electrophoresis sample buffer containing 62.5 mM Tris-HCl, pH 6.8, 2.3% SDS, 10% glycerol, 5%  $\beta$ -mercaptoethanol and 0.1% bromophenol blue for 5 min and then centrifuging in an Eppendorf microfuge. Supernatants were subjected to SDS PAGE as previously described.

**Western Blotting:** Western blotting was done essentially according to Towbin et al. (15). Briefly, gel was equilibrated in blotting buffer containing 150 mM glycine, 20 mM Tris-base and 20% methanol for 30 min after second-dimension electrophoresis. Electrotransfer of proteins from gel to nitrocellulose membrane (Schleicher & Schuell, Inc., Keene, NH) was carried out in the same solution for 90 min at 100 V. Subsequently, the nitrocellulose membrane was washed in blocking buffer containing 1% gelatin, 150 mM NaCl, 8 mM Tris-HCl and 0.6 mM Tris-base for 1 h and incubated with mouse monoclonal anti rat  $\alpha$ -casein antibody in fresh blocking buffer overnight at room temperature. Excess antibody was removed by washing in blocking buffer plus 0.05% Tween-20. The nitrocellulose was then incubated with normal goat serum for 30 min and then with peroxidase-conjugated goat anti-mouse IgG antibody for 2 h. After incubation with goat antiserum, the nitrocellulose membrane was washed several times in 150 mM NaCl, 8 mM Tris-HCl, 0.6 mM Tris base and 0.05% Tween 20, and the peroxidase enzyme was detected by staining with amino ethylcarbazole and hydrogen peroxide. The stained nitrocellulose membrane was washed in water, air-dried, and exposed to Kodak XAR-5 film for autoradiography.

## RESULTS

### Analysis of Skim Milk Proteins

To obtain an *in vivo* reference for the culture studies, mouse milk was obtained as described in the Materials and Methods, and the proteins were resolved by two-dimensional gel electrophoresis. The characteristic positions of the 44,000-, 41,000-, 26,000-, 24,000-, and 20,000-mol-wt proteins (Fig. 1) correspond to the positions of the five caseins, as demonstrated by comparison with the positions of purified casein polypeptides resolved by two-dimensional gel electrophoresis.

These are designated  $\alpha_1$ ,  $\alpha_2$ ,  $\beta$ ,  $\gamma$ , and  $\delta$  according to the nomenclature of Hennighausen and Sippel (16), as opposed to the earlier nomenclatures (17, 18). The protein designated "a" corresponds to one of the major whey proteins, albumin (19). Its identity was verified by immunoprecipitation with antialbumin antibody (Fig. 2A).

The protein with the molecular weight of 14,000 is whey acidic protein (WAP). It is known that WAP contains methionine but does not contain tyrosine (20). In our experiments, the 14,000-mol-wt protein could be labeled using [ $^{35}$ S]methionine, but could not be detected when [ $^3$ H]tyrosine was used (data not shown). In rat milk, WAP occurs in three forms with different degrees of phosphorylation (21). But, in mouse, only one form has been reported and it may not be a phosphoprotein (20). The spot beneath WAP is  $\alpha$ -lactalbumin as judged by its molecular weight (22) and by our ability to immunoprecipitate it with antiserum to  $\alpha$ -lactalbumin (Fig. 2C).

The 80,000-mol-wt protein is transferrin, based on immunoprecipitation with anti-mouse transferrin antibody (Fig. 2B) and two-dimensional gel electrophoresis of mixtures of cold purified transferrin with radiolabeled medium from radiolabeled cultured mammary epithelial cells (data not shown).

To make a direct comparison with the  $^{35}$ S-labeled secreted proteins in culture, mice were injected with [ $^{35}$ S]methionine and milked as described in Materials and Methods. The two-dimensional gel pattern of the labeled milk proteins was similar to that of the cold milk, with a few exceptions (Fig. 3), namely that the intensity of the albumin spot was much lower with labeled milk, and two spots with molecular weights  $\sim$ 12,000 (d in Fig. 3) were spots in radiolabeled but not in cold milk. The spot at the basic end of WAP probably is not a modified form of the molecule as discussed above.



FIGURE 1 Two-dimensional gel electrophoresis of mouse skim milk. 300  $\mu$ g of milk protein was precipitated with trichloroacetic acid, washed with acetone, and dissolved in lysis buffer containing 9.5 M urea, 2.0% NP-40, 5%  $\beta$ -mercaptoethanol, 1.6% pH 5-7, 0.4% pH 3.5-10 ampholine. First dimension was nonequilibrium pH gradient electrophoresis with acidic end on left and basic end on right. Second dimension was SDS 6-15% PAGE. The gel was stained with Coomassie Blue to visualize the spots. a, albumin;  $\alpha_1c$ ,  $\alpha_2c$ ,  $\beta c$ ,  $\gamma c$ , and  $\delta c$ ,  $\alpha_1$ -casein,  $\alpha_2$ -casein,  $\beta$ -casein,  $\gamma$ -casein, and  $\delta$ -casein, respectively; W, whey acidic protein; al,  $\alpha$ -lactalbumin; b, an 80,000-mol-wt protein, most probably transferrin.

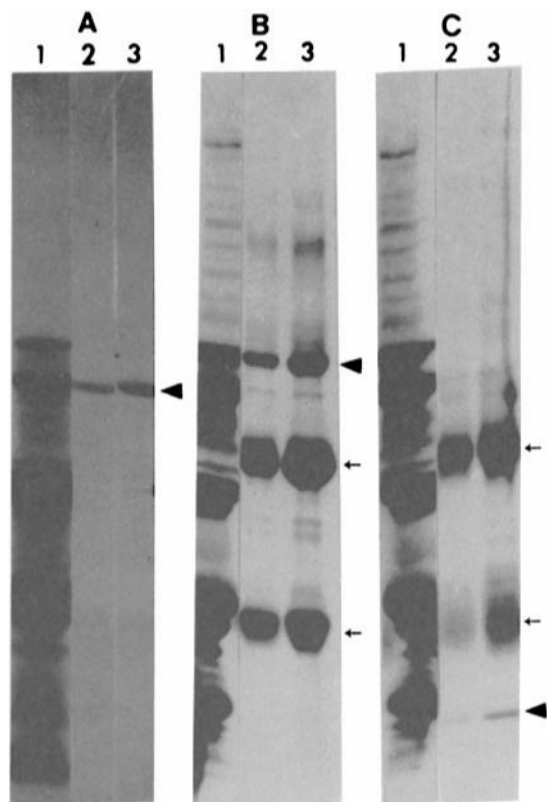


FIGURE 2 Identification of albumin, transferrin, and  $\alpha$ -lactalbumin by immunoprecipitation and one-dimensional SDS PAGE. (A) Autoradiograms of [ $^{35}$ S]methionine-labeled skim milk proteins. Lane 1, total skim milk proteins; lane 2, immunoprecipitated proteins after incubating radiolabeled skim milk with 6  $\mu$ l of anti-mouse albumin antiserum; lane 3, same as 2, but with 18  $\mu$ l of antiserum. (B) Coomassie-Blue-stained gels of: lane 1, 150  $\mu$ g of total skim milk proteins; lane 2, proteins precipitated with 10  $\mu$ l of anti-mouse transferrin antiserum; lane 3, same as 2 but with 20  $\mu$ l of antiserum. (C) Coomassie-Blue-stained gels of: lane 1, 150  $\mu$ g of total skim milk proteins; lane 2, proteins precipitated with 6  $\mu$ l of anti-rat  $\alpha$ -lactalbumin antiserum; lane 3, same as 2 but with 25  $\mu$ l of antiserum. The large arrows point to the specific proteins immunoprecipitated while the small arrows denote heavy and light chains of the immunoglobulins.

### Secretion of Proteins from Freshly Isolated Cells

The mammary gland is composed of myoepithelial cells, fibroblasts, and adipocytes, in addition to epithelial cells. While the epithelial cells are the source of most of the milk proteins (except for albumin), their secretory activity may be influenced by one or more of the other cell types (for example, see reference 23). It was therefore important to determine which proteins were being secreted by freshly isolated cells so that a profile of the initial activity of the epithelial cells alone (or by themselves) could be established and used as a basis to study cells in culture. As is clear from Fig. 4, freshly isolated cells secreted all the major proteins observed in radiolabeled milk, except for albumin. The similarities between Figs. 4, and 1, and 3 enabled us to make clear assignments as to the identity of most of the spots of Fig. 4. Spots *b'* and *b* in Fig. 4 were shown further to be identical by peptide mapping after digestion with V8 protease (data not shown). The absence of albumin in the medium is expected since mammary epithelial cells do not synthesize this protein, and it appears in milk

only after being sequestered from the blood stream (24). In addition to secreting all other proteins that are found in skim milk, the isolated cells secreted some additional proteins, notably a 67,000-mol-wt protein (Fig. 4, *f*), which is most probably butyrophilin, a major protein of the milk fat globule membrane (25). This was identified as such on the basis of comparison with two-dimensional gel patterns of purified milk fat globule membranes (data not shown). The *e* proteins are a series of proteins with molecular weight  $\sim$ 20,000 and appear to be minor proteins in some milk preparation (for example, see Fig. 6*A*). The *z* protein and several other unmarked minor proteins are not found in skim milk.

The possibility that some of these "extra" secreted proteins (e.g., *z* protein) were produced by contaminating nonepithelial cells is unlikely. Cells were separated on a Percoll-gradient, the secreted proteins were analyzed by two-dimensional gel electrophoresis, and the patterns were compared with that described above. There were no significant differences between the two patterns, demonstrating that the "extra" secreted proteins were of epithelial (or myoepithelial) origin (data not shown; see also below).

### Analysis of Proteins Secreted by Cultured Cells

When epithelial cells were placed in tissue culture plastic dishes at high density for 1 wk, the pattern of secreted proteins was significantly different from that of proteins in milk (Fig. 5*A*). The  $\alpha_1$ -,  $\alpha_2$ -,  $\beta$ -,  $\gamma$ -, and  $\delta$ -caseins were absent, as were the whey acidic protein and  $\alpha$ -lactalbumin. However, the 80,000-mol-wt protein was present, albeit in reduced amounts, as was the 67,000-mol-wt protein. Many new proteins were also secreted, including a 140,000-mol-wt protein (Fig. 5, *g*) and proteins marked X and Y. The ratio of *e* proteins to other proteins was significantly elevated. Cells plated onto collagen gels secreted many of the proteins that were secreted by cells on plastic but also produced additional proteins (Fig. 5*B*). Some of the caseins ( $\alpha$  and  $\beta$ ) were now secreted at low levels, while levels of the 80,000-mol-wt protein and butyrophilin were significantly increased. Finally, cells that were plated onto gels that were subsequently released from the dish produced and secreted considerable quantities of  $\alpha_1$ -,  $\alpha_2$ -, and  $\beta$ - and  $\gamma$ -caseins, as well as slightly increased levels of 80,000-mol-wt protein (Fig. 5*C*). In addition, these cells produced most of the proteins that were produced by cells on attached gels. It should be noted that only a small quantity of whey acidic protein was detected in culture media from cells on floating gels and virtually none on any of other substrata.

The identity of various caseins and other proteins secreted from cultured cells was confirmed by two-dimensional gel electrophoresis of a mixture of cold skim milk and [ $^{35}$ S]-methionine-labeled medium from a culture of cells on a floating gel. Fig. 6*A* shows the Coomassie Blue staining of the gel, and Fig. 6*A'* shows the autoradiography of the same gel. The 80,000-mol-wt protein,  $\alpha_1$ -,  $\alpha_2$ -,  $\beta$ -, and  $\gamma$ -casein and *e* proteins from skim milk and culture medium ran coincidentally to each other. The identity of spots of  $\alpha_1$ - and  $\alpha_2$ -casein in the culture medium was further confirmed by western blotting. Fig. 6*B* shows the autoradiogram of the nitrocellulose membrane after the proteins were electrotransferred. Most of the proteins were efficiently transferred from the polyacrylamide gel to the nitrocellulose membrane. Fig. 6*B'*

FIGURE 3 Fluorograph of a two-dimensional gel of [<sup>35</sup>S]methionine-labeled skim milk proteins. 2 mCi of [<sup>35</sup>S]methionine was peritoneally injected into three lactating mice. Milk was collected 12 h later and processed as described in Fig. 1. The sample loaded contained  $2.5 \times 10^5$  dpm, and the gel was exposed to x-ray film for 4 d. For symbols' meaning, see the legend for Fig. 1. *d*, a 12,000-mol-wt protein.

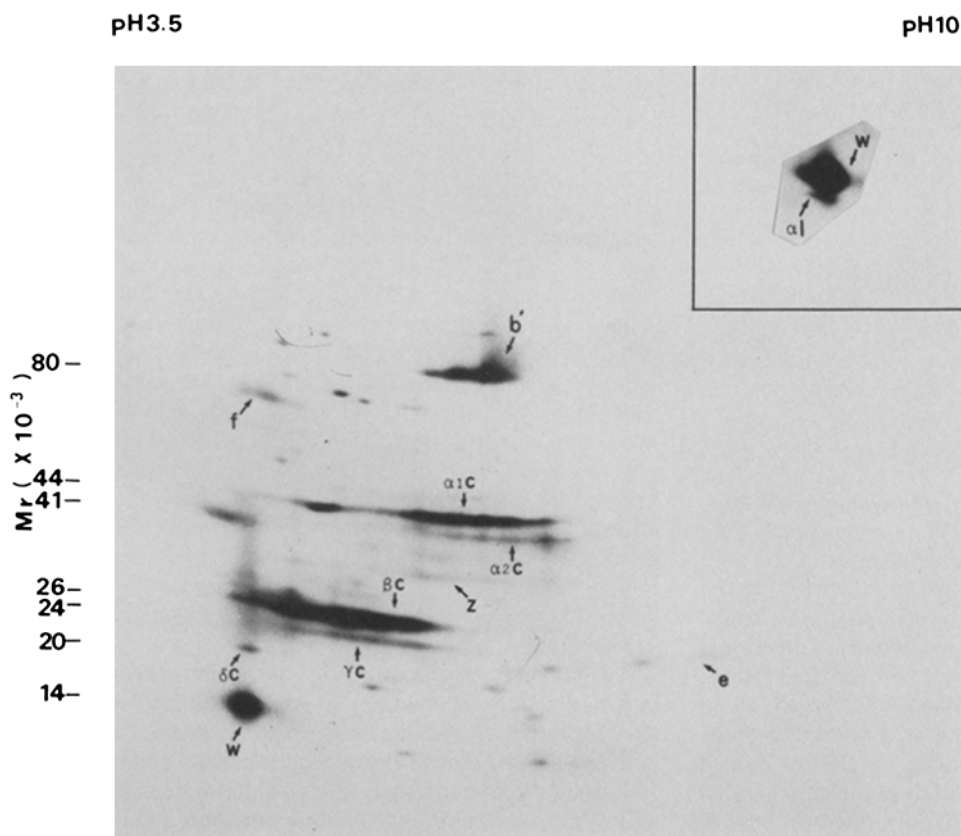
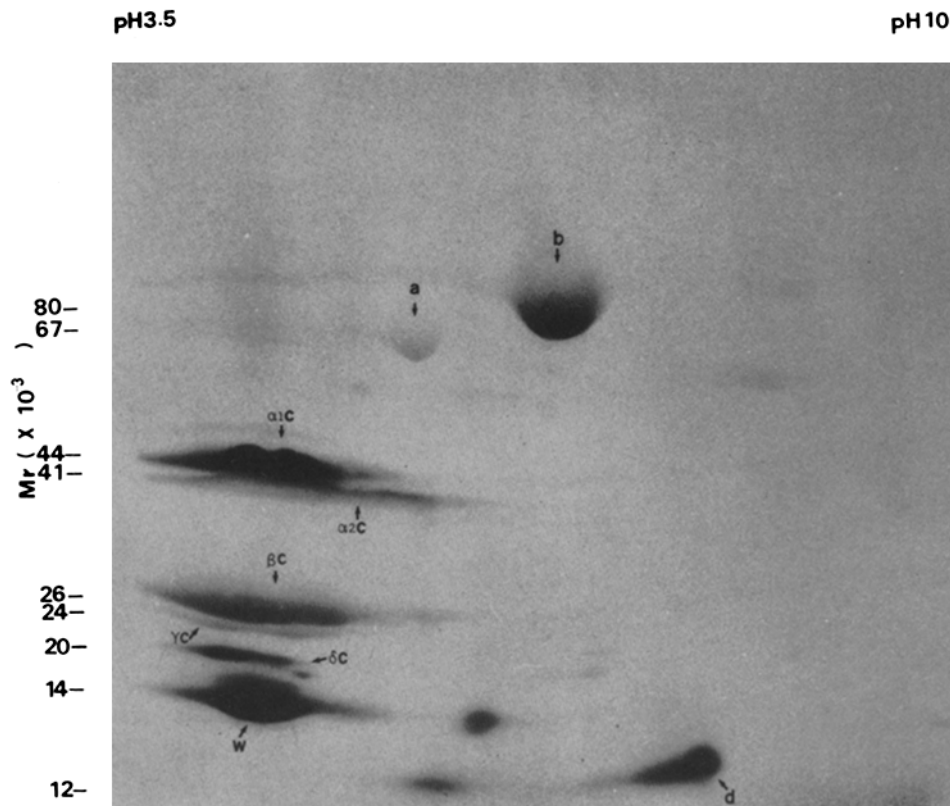


FIGURE 4 Fluorograph of a two-dimensional gel of [<sup>35</sup>S]methionine-labeled proteins secreted by freshly isolated epithelial cells.  $3 \times 10^6$  fresh isolated cells (all three hormones are present during the whole isolation procedure) were labeled with 100  $\mu$ Ci of [<sup>35</sup>S]methionine in suspension in methionine-free medium. Medium was collected after 2-h labeling. The sample loaded contained  $1.6 \times 10^5$  dpm, and the gel was exposed to x-ray film for 1 wk. Symbols as defined in the legend for Fig. 1. *f*, a 67,000-mol-wt protein, possibly butyrophilin, the major fat globule membrane protein. *b'*, shown to be the same as the 80,000-mol-wt protein found in milk. *e*, series of proteins with 20,000 mol wt. The *e* proteins are a minor component in some preparations of mouse skim milk (see Fig. 6A). *z*, a protein not seen in skim milk. (Inset)  $\alpha$ -lactalbumin after longer exposure.

shows the peroxidase staining after immunoreaction with monoclonal anti- $\alpha$ -casein antibody, demonstrating that the two spots correspond to  $\alpha_1$ - and  $\alpha_2$ -casein. Cross-reactivity of different monoclonal  $\alpha$ -casein antibodies to  $\alpha_1$ - and  $\alpha_2$ -casein has been demonstrated before (14a).

It is noticeable that caseins secreted from freshly isolated or cultured epithelial cells ran slightly differently than caseins in skim milk, in that they moved more toward the basic end (compare Fig. 6A' and B where the same sample was run; also compare Figs. 4 and 5C, with Figs. 1 and 3, and notice

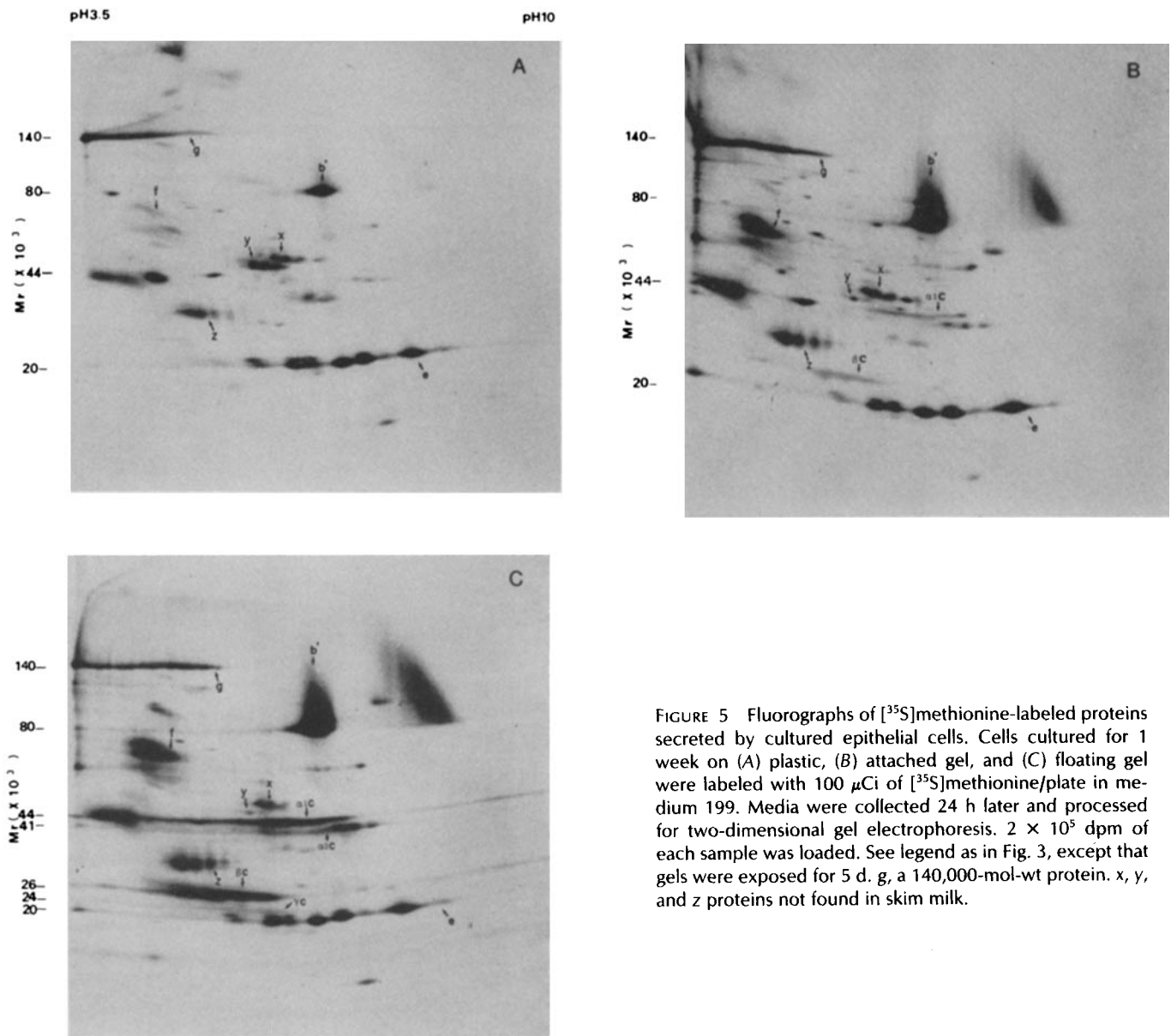


FIGURE 5 Fluorographs of [ $^{35}\text{S}$ ]methionine-labeled proteins secreted by cultured epithelial cells. Cells cultured for 1 week on (A) plastic, (B) attached gel, and (C) floating gel were labeled with 100  $\mu\text{Ci}$  of [ $^{35}\text{S}$ ]methionine/plate in medium 199. Media were collected 24 h later and processed for two-dimensional gel electrophoresis.  $2 \times 10^5$  dpm of each sample was loaded. See legend as in Fig. 3, except that gels were exposed for 5 d. g, a 140,000-mol-wt protein. x, y, and z proteins not found in skim milk.

relative positions of caseins to 80,000-mol-wt spot). It is possible that this charge difference may be due to differences in the degree of phosphorylation between caseins in skim milk and in the medium of isolated epithelial cells. It is surprising, however, that when the skim milk and radiolabeled medium samples were mixed, both sets of caseins migrated to the same positions (Fig. 6, A and A'). The cause of this remains to be determined, but may be due to differences in the stability of phosphate groups in the separate samples.

To quantitate some of the changes in the levels of secreted proteins, spots corresponding to the 80,000-mol-wt protein,  $\alpha_1$ -,  $\alpha_2$ -,  $\beta$ -, and  $\gamma$ -caseins and the 20,000-mol-wt (e in Fig. 7) protein series were cut out and counted and the data plotted as shown in Fig. 7. Clearly, the levels of the (Fig. 7, e) proteins varied little with the nature of the substrata while the caseins were secreted at significant levels only by cells on released gels. In contrast, the 80,000-mol-wt protein was secreted in substantial quantities by cells on attached collagen gels, and

its level was increased only approximately 1.5-fold upon flotation of the gels.

#### Examination of Cultures for Contaminating Fibroblasts

As some proteins were produced and secreted by the cells on all three substrata and as the same proteins were absent from milk (e.g., x, y, and z proteins), we considered the possibility that these proteins might not be produced by epithelial cells but might be produced by fibroblast contamination. This was ruled out for the following reasons: Firstly, when mammary fibroblasts were isolated from the gland and cultured, their morphology was clearly different from that of epithelial cultures (Fig. 8). Secondly, when mammary fibroblast cultures were radiolabeled with [ $^{35}\text{S}$ ]methionine and the secreted proteins were analyzed (Fig. 9), the proteins were distinct from those found in cultures of epithelial cells (compare with Figs. 5).

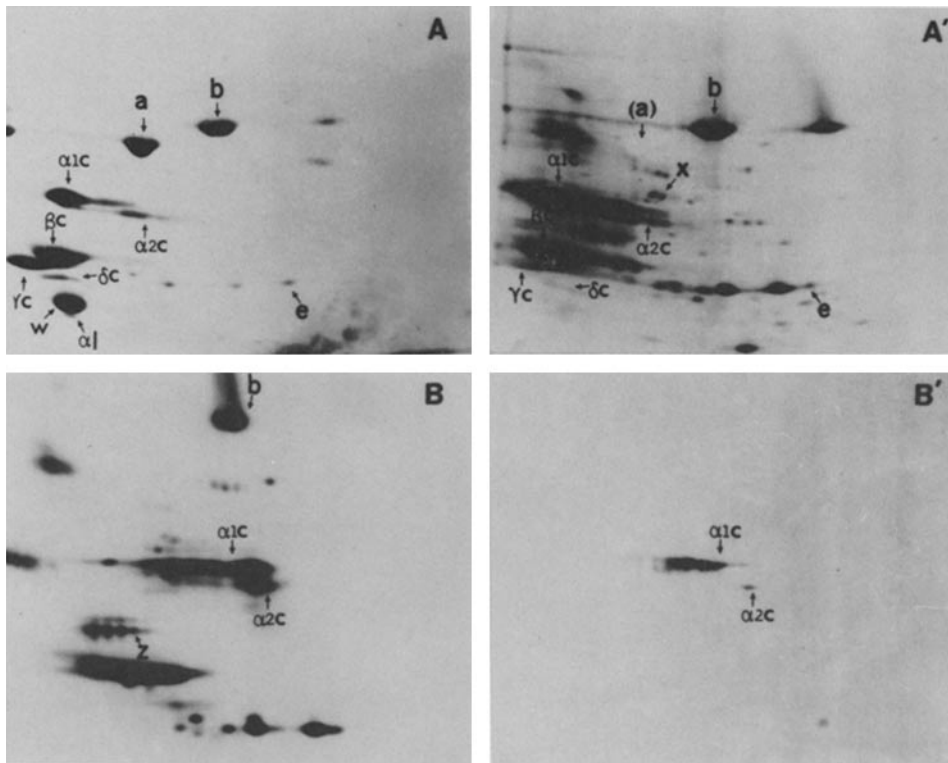


FIGURE 6 Identification of medium proteins secreted from cells cultured on floating gels. Sample containing  $1.5 \times 10^5$  dpm counts was mixed with  $300 \mu\text{g}$  of cold skim milk proteins. (A) Coomassie Blue staining shows the major proteins in skim milk. (A') autoradiography of the same gel shown in A. a in A' marks the position of cold albumin. (B and B') Western blotting of the same sample as in A'. (B) autoradiography of the electrotransferred proteins. (B') Peroxidase staining after immunoreaction with monoclonal anti  $\alpha$ -casein antibody. The two spots are  $\alpha_1$ -casein and  $\alpha_2$ -casein.

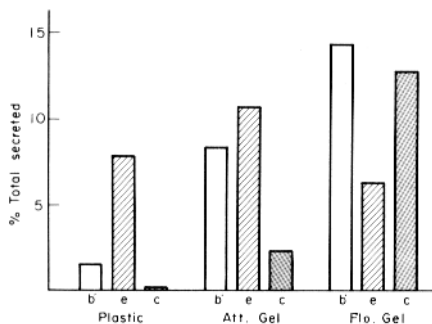


FIGURE 7 Quantitation of radioactivity incorporated into gel spots b', e, and c, as referred to in Fig. 5. c includes all four caseins; e all spots in 20,000-mol-wt series, and b', the 80,000-mol-wt protein. Spots were cut from two-dimensional gel of medium sample of plastic, attached and floating gel cultures.

### Protein Secretion on Glutaraldehyde Modified Gels

The floating collagen gel provides two features for the cultured epithelial cells that are not provided for by the attached gel: (a) it makes available the basal surface of the cells for uptake of nutrients, and (b) it provides a flexible support which can be contracted by the cells. Gel contraction may have several consequences, including a change in the shape of the cells from cuboidal to columnar (3). To assess the relative importance of these two parameters, we cross-linked collagen gels with glutaraldehyde as described in Materials and Methods. These gels could be released from the culture dish and made to float but did not contract appreciably. The analysis of the [ $^{35}\text{S}$ ]methionine-labeled secreted proteins revealed that the cells failed to produce any of the five caseins whether the glutaraldehyde-treated gel was attached (Fig. 10A) or floating (Fig. 10B). In fact, the pattern of secretory proteins was very similar to that of the set of proteins produced on native, attached gels (compare Figs. 10 and 5B).

The experiment thus showed that gel contraction or some consequence thereof is necessary for coordinate synthesis and secretion of caseins and that making the basolateral surface of the cells available for nutrient uptake is not sufficient by itself.

### Is the Regulation by Substratum at the Level of Secretion, Synthesis, or Stability?

It is possible that the extracellular matrix components exert their modulating effect on casein secretion by regulating the production and/or stability of the caseins. An alternative possibility is that caseins are synthesized on all substrata but that their release from the cells to the medium occurs only in cultures on floating gels. To test which of these possibilities is correct, we examined the intracellular and media caseins in epithelial cells cultured on different substrata (Fig. 11). When cells were labeled for 12 h with [ $^{35}\text{S}$ ]methionine, medium from cells cultured on floating gels contained a considerable quantity of  $\alpha_1$ -casein, and only a very small amount was found inside the cell (Fig. 11B). When cells cultured on plastic were examined (Fig. 11A), only the same low level of casein was found inside the cells, demonstrating that caseins secreted by floating gel cultures had not simply accumulated intracellularly in cells that were on plastic substrata.

### DISCUSSION

That the mammary gland from the late pregnant animal has the information and the capability to mimic the lactating state if suitable hormones are present was shown by organ culture studies in which addition of prolactin caused an accumulation of casein mRNA and the synthesis and secretion of caseins (26, 27). This ability to produce differentiated products becomes lost when epithelial cells are removed from the late pregnant gland and plated on plastic culture dishes in the presence of lactogenic hormones (3, 4). However, Emerman and colleagues (3, 4) discovered that if these cells were plated



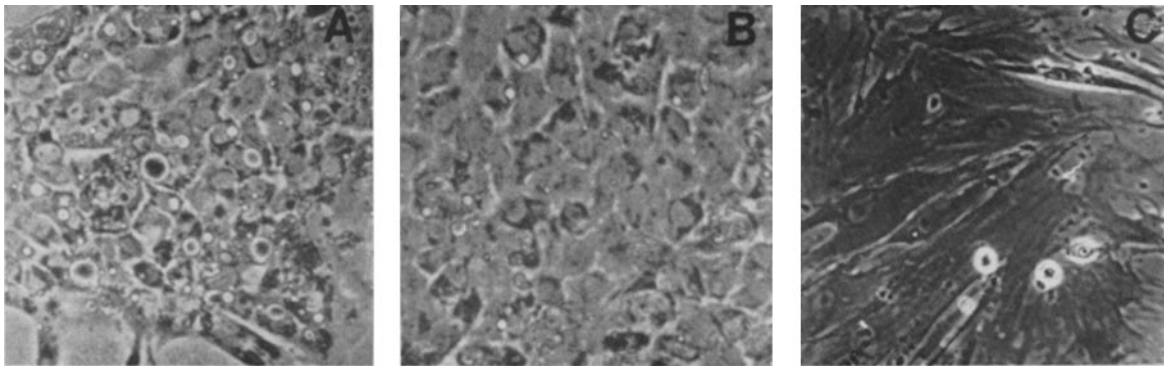


FIGURE 8 Phase-contrast micrographs of cultures of mammary epithelial and fibroblastic cells. (A) epithelial cells cultured on tissue culture plastic. (B) Epithelial cells cultured on an attached collagen gel. (C) Fibroblasts cultured on tissue culture plastic dishes.  $\times 250$ .

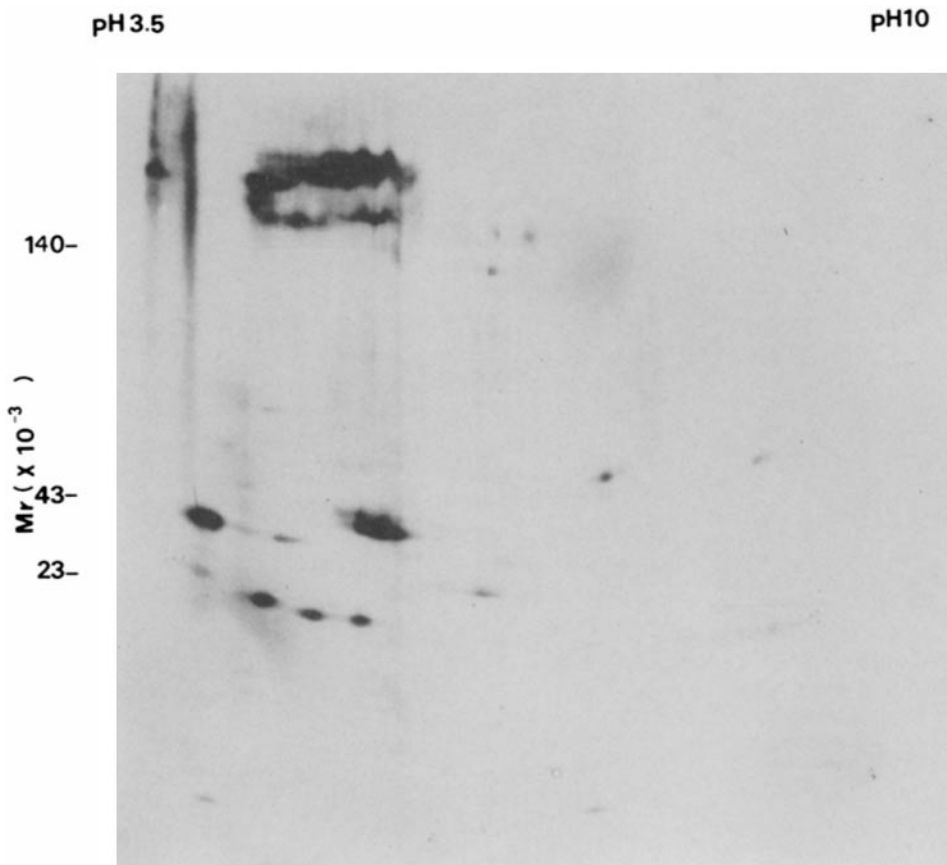


FIGURE 9 Fluorograph of a two-dimensional gel of [ $^{35}\text{S}$ ]methionine-labeled proteins secreted into the medium of cultured mammary fibroblasts. Confluent fibroblast culture established as described in the Materials and Methods was labeled with 100  $\mu\text{Ci}$  of [ $^{35}\text{S}$ ]methionine. After 24 h, medium was collected and processed for 2-D gel electrophoresis.

onto collagen gels that were then allowed to float, they developed a distinctive secretory morphology and secreted  $\gamma$ -caseins.

The results reported in this paper provide a picture of the full extent to which changes in the substrata can influence the expression of tissue specific functions. Our principal findings are:

(a) The nature and the physical state of the substratum can regulate the expression of many proteins that are present in milk. These include  $\alpha_1$ -,  $\alpha_2$ -,  $\beta$ -, and  $\gamma$ -caseins, an 80,000-mol-wt protein that is probably transferrin, and a 67,000-mol-wt protein that is probably butyrophilin, a component of the milk fat globule membrane. Whey acidic proteins and  $\alpha$ -lactalbumin are not produced in significant quantities in 7-d cultures.

(b) There appears to be a degree of coordinate control over synthesis and secretion of  $\alpha_1$ -,  $\alpha_2$ -,  $\beta$ -, and  $\gamma$ -caseins. These are all altered to a similar extent by changes in the composition and physical structure of the substratum.

(c) Secretion of the 67,000- and 80,000-mol-wt protein is also modulated by the substratum but in a different way from the secretion of  $\alpha_1$ -,  $\alpha_2$ -,  $\beta$ -, and  $\gamma$ -caseins. For example, on attached collagen gels the 80,000-mol-wt protein and the 67,000-mol-wt protein are produced in substantial quantities, while none of the caseins are expressed at a high level. When the gel is floated, there is an increase in the levels of the 80,000-mol-wt protein in the medium but a much greater proportional increase in the levels of all of the caseins.

(d) The substrata and the culture conditions employed in these experiments are not adequate to maintain whey acidic

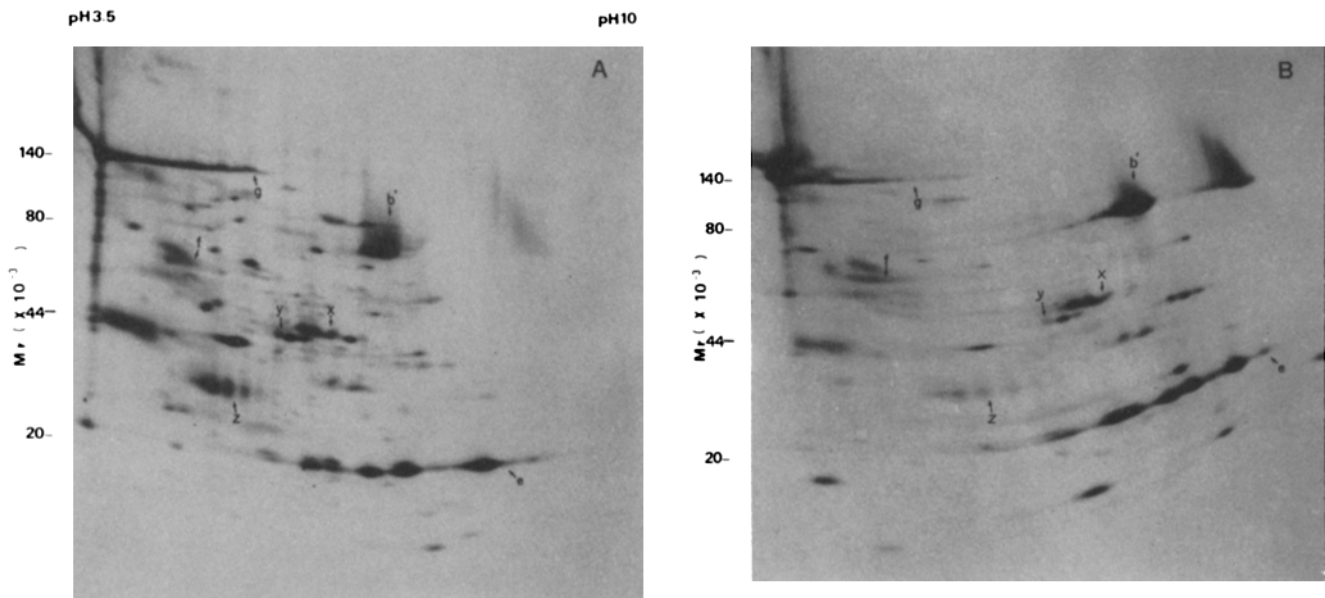


FIGURE 10 Fluorographs of two-dimensional gels of [ $^{35}\text{S}$ ]methionine-labeled proteins secreted into the culture medium by cells cultured for 1 wk on (A) attached glutaraldehyde cross-linked and (B) floating glutaraldehyde cross-linked gels. Legend as in Fig. 5. The cross-linked gels were prepared as described in the Materials and Methods.

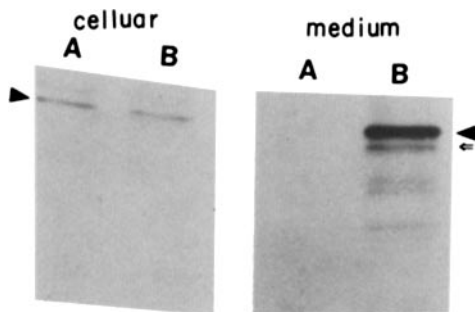


FIGURE 11 Immunoprecipitation of intracellular and secreted caseins of cultured epithelial cells. Cells on plastic and floating gels were labeled with  $100\ \mu\text{Ci}$  of  $^{35}\text{S}$ -methionine. After 12 h, media were collected. Cells were washed with cold phosphate-buffered saline buffer and then lysed in lysis buffer containing 0.2% NP-40 and 0.5% deoxycholate. Half of the media and cell lysate samples were then immunoprecipitated with anti- $\alpha_1$ -casein antibody and electrophoresed on 10% polyacrylamide gel. (A) Culture on plastic dish. (B) Culture on floating gel. The arrowhead points to the  $\alpha_1$ -casein and small arrow points to  $\alpha_2$ -casein. The faint bands underneath are probably degradation products of caseins.

protein and  $\alpha$ -lactalbumin levels at their *in vivo* levels. Freshly isolated cells secrete considerable quantities of whey acidic protein (Fig. 4) but, except for a small amount in floating gel cultures, virtually none could be detected in the media of any of the cultures. Our preliminary results indicated that after 1 mo in culture the profile of proteins secreted by cells on floating gels was similar to that of 7-d cultures, except that a significant amount of WAP was also secreted (Lee, Parry, and Bissell, unpublished observations). Thus, WAP induction may require other factors that are present only in long-term culture.  $\alpha$ -Lactalbumin is a minor whey protein of mouse milk and while it is produced in small amounts by freshly isolated cells it is not detected in any of the culture media. Control of whey acidic protein and  $\alpha$ -lactalbumin production is thus quite definitely independent of control of  $\alpha_1$ -,  $\alpha_2$ -,  $\beta$ -, and  $\gamma$ -caseins and of the 80,000- and 67,000-mol-wt protein. As demonstrated in rat, whey acidic protein is induced mainly

by hydrocortisone while casein is regulated mainly by prolactin (28). Thus the hormonal regulation and optimum culture condition for the expression of whey acidic proteins and caseins are quite different. Our observations on  $\alpha$ -lactalbumin production are consistent with our earlier measurements of lactose production by cultured cells. Typically, cells obtained from late pregnant mice produced only a small quantity of lactose in comparison to that produced by the lactating gland (6). Ono and Oka (29) have shown in explant cultures that the accumulation of  $\alpha$ -lactalbumin is inhibited by high concentrations of hydrocortisone. While it is conceivable that the hydrocortisone concentration used in our studies was not optimal for  $\alpha$ -lactalbumin production, the same hydrocortisone levels were used for our experiments with freshly isolated cells when  $\alpha$ -lactalbumin was produced (Fig. 4).

(e) Cultured cells secrete many proteins that are not found in milk, and many of these are secreted independently of the substratum. We have demonstrated that these proteins are not secreted by contaminating cells such as fibroblasts. However, the possibility that myoepithelial cells may be the source of these additional proteins has not been ruled out. The functions of these proteins are not understood at the present time. It is possible that they are produced by epithelial cells *in vivo* but are secreted basally rather than apically in the milk, while in culture either the cells fail to maintain aspects of their polarity or the proteins are secreted into the basal layer and then diffuse into the culture medium. Another possibility is that, despite the fact that cells can produce tissue-specific milk proteins on a suitable substratum, the culture environment fails to prevent (or in fact enhances) the production of nonmilk proteins. The 140,000-mol-wt protein, which is secreted in large quantities independently of the substratum, is not found in skim milk, but our preliminary data suggest that it may be a component of the milk fat globule membrane. However, further characterization is needed, and this protein may also prove to be a major culture-induced component.

One important conclusion to be reached from these findings is that the substratum control of the cellular differentiation program does not occur in an "on-or-off" fashion. The sub-

stratum regulates the production of most of the milk proteins by a distinct mechanism, and does not modulate the levels of all milk components in concert with each other.

A significant question to ask in this experimental system is how the extracellular matrix regulates the production of milk specific proteins? We have shown in this paper that control of secretion is not the regulatory step in that no elevated intracellular levels of caseins are found in cells on plastic. Recent preliminary data by Supowit et al. (30) have demonstrated that the level of mRNA for casein was greater in cells on floating gels than in cells on attached gels and that prolactin induced a further increase in mRNA levels. Thus, transcriptional control of mRNA may be operating. Regulation of the degradation of newly synthesized proteins may also be important. Razooki Hasan et al. (31) have recently demonstrated in organ culture that considerable quantities of newly synthesized caseins are rapidly degraded during the course of secretion. It will be important to assess the rates of degradation on the various culture substrata employed in these experiments.

The role of the floating collagen gel in the process of regulation of milk-specific proteins is intriguing. By use of glutaraldehyde cross-linked gels, we demonstrate here that the gel is not a simple floating permeable platform. Contraction of the gel, brought about by cellular activity, appears to be important. The consequences of contraction are considerable, one of the most obvious being a change in cell shape (32), although changes in the organization of the cytoplasm are equally possible (2). In considering mechanisms regulating tissue specific protein synthesis, it is important to realize that the interaction between the substratum and the cells may not be direct as discussed previously (2). The gel may allow the cells to produce their own extracellular matrix components that deposit as a basal lamina between the cells and the gel. It is quite possible that the true inducing structure is not the gel itself but is instead the cell's own basal lamina. Electron microscopic studies have shown a distinct basal lamina beneath cells on floating gels but have failed to find a basal lamina beneath cells on plastic (32).

In preliminary experiments, we recently analyzed the extracellular matrix proteins produced by cells on plastic surfaces, attached, and floating collagen gels. We find distinct sets of proteins deposited according to the substratum used for culturing (33). In particular, we find an increased proportion of type-IV collagen produced by the cells on collagen gels as well as differences in the relative proportions of various glycosaminoglycans (34). We are currently investigating the role of these cell-synthesized molecules in the cell-substratum interactions discussed here.

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Note Added in Proof: Recently, Ray et al. reported that long-term

cultures of rat mammary epithelial cells produced and secreted some casein even when maintained on plastic dishes (35). All our experiments reported here were short-term cultures.

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