

## Isolation and characterization of new proteins produced by the infusion of colchicine in goat mammary gland

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Three new proteins have now been isolated from goat milk obtained after colchicine is infused into the mammary gland. Two of the proteins are proline-rich, and a third is a very acidic phosphoprotein. One of the proline-rich proteins is related compositionally to a sheep colostrum proline-rich protein, which has been shown to have a regulatory effect on the immune response (Janusz, M., Stavoscik, K., Zimecki, M., Wieczorek, Z., and Lisowski, J. (1981) *Biochem. J.* 199, 9-15). Other aspects of colchicine-treated milks are described.

### Introduction

Drug treatment of secretory tissues produces changes in the patterns of proteins normally produced: isoproterenol treatment causes a dramatic increase in the proline-rich protein content of salivary secretions of rats [1]; androgen treatment increases the release of prostate protein, including a proline-rich protein [2]; and colchicine alters the secretory patterns of bile proteins [3]. In the former two cases, drug treatment causes the release of proline-rich protein and other proteins which are normally secreted, but the proline-rich protein content increases more dramatically.

The plant alkaloid, colchicine, interacts with the microtubule protein, tubulin, and inhibits mitosis [4]; it also affects a number of secretory processes, including suppression of milk secretion [5-7]. Studies on the biochemical and ultrastructural ef-

fects of colchicine on rat and cow mammary epithelia suggest that both intracellular transport and secretion of milk constituents depend upon an intact microtubule system [8].

Patton [5,6] showed that the suppression of lactation produced by infusion of colchicine into the goat udder is reversible and that although the volume of milk was reduced, the major components of milk from infused udders were essentially normal. However, minor differences in the proteins were found by polyacrylamide gel electrophoresis. This study on the isolation and characterization of alterations in milk proteins produced by infusion of colchicine was initiated to learn more about the effects of this drug on the production and transport of milk proteins.

### Experimental

**Starting material.** Two goats in their 6th month of lactation received 5 mg colchicine by infusion in one teat canal after the p.m. milking on two successive days. The other teat was used as a control. The milk samples were taken at the usual

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milking time on the following morning, and stored frozen for a month before they were lyophilized. About 200 ml of control samples were obtained while the colchicine-treated samples were reduced to about 100 ml. To separate the fat, casein and whey fractions, 3 g of lyophilized goat milk were added to 25 ml water, stirred, centrifuged, and cooled in a freezer so the fat pellet could be removed. The skim milk was then warmed to 25°C and adjusted to pH 4.2 with 1 M HCl. The precipitated casein was recovered by centrifugation, washed two times with 6 ml water, and readjusted each time to pH 4.2. The casein wash was added to the whey fraction, which was dialyzed at 3°C. On dialysis of the whey fraction, a small amount of precipitate was isolated by centrifugation. Both the whey and casein fractions were recovered by lyophilization.

**Fractionation.** Control and colchicine-treated samples of casein were each chromatographed on a microgranular DEAE-cellulose column 2 × 35 cm, equilibrated with 0.01 M imidazole-HCl buffer, pH 7.0, at 3°C. The proteins were eluted with a linear gradient of (1) 250 ml 0.01 M imidazole-HCl, pH 7.0, and (2) 250 ml 0.01 M imidazole-HCl/1.0 M NaCl, pH 7.0, followed by buffer (2) made to 4 M urea. Certain fractions from the casein of the colchicine-treated udder eluted from DEAE-cellulose contained the novel proteins designated I-, II- and III-proteins and were fractionated further.

**I-fraction.** The I-fraction was applied to a Sephadex G-50 \* superfine column 0.9 × 163 cm, 3°C, equilibrated with 0.01 M NH<sub>4</sub>HCO<sub>3</sub>. Its elution profile showed a symmetrical peak with little fractionation. A fraction representing the center portion of the peak, 10 mg, was chromatographed on a CM-Sephadex G-25 column 0.9 × 45 cm, equilibrated with 0.05 M potassium phosphate, pH 5.0, 3°C. With the starting buffer, 3.2 mg of material designated I-1 protein was recovered. A second fraction, 5.5 mg, designated I-2 was eluted with higher potassium chloride concentration and pH of 6.2.

**II-fraction.** The II-fraction was applied to a Sephadex G-50 superfine column, 0.9 × 163 cm at

3°C, equilibrated with 0.01 M NH<sub>4</sub>HCO<sub>3</sub>. Although the elution peak was symmetrical, the protein in the trailing edge of the peak (II-protein) was used for further studies, because disc-gel electrophoresis showed that it contained a single zone compared to two for the leading edge.

**III-fraction.** The III-fraction, 175 mg, contained casein in addition to the III-protein. It was dissolved in 10 ml water by titrating to pH 7.6 with 0.1 N NaOH. The casein was precipitated at pH 4.2 with 0.1 M HCl, leaving much of the III-protein, 45 mg, in the supernatant. This fraction was applied to a Bio-Gel P 100, 100–200 mesh column 2 × 63 cm, 3°C, equilibrated with 0.01 M NH<sub>4</sub>HCO<sub>3</sub>. The eluted fractions, III-1, III-2 and III-3, contain 7.5, 5.0 and 11.0 mg protein. Another peak eluted later apparently contains smaller peptides.

**Gel electrophoresis.** Slab gel electrophoresis in the presence of urea at pH 3 was carried out as previously described [9]. Discontinuous SDS-gel electrophoresis was by the method of Laemmli [10]. The method of Weber and Osborn [11] was also used; all molecular weights were calculated based on the latter method. Disc-gel polyacrylamide electrophoresis was according to Davis [12] at pH 8.6, but in 4 M urea. Disc-gel electrophoresis (pH 4.3) in 8 M urea was a modification of the method of Reisfeld et al. [13].

**Immunodiffusion.** Ouchterlony double diffusion was carried out in 1% agar in phosphate-buffered saline, pH 7.2 [14].

**Phosphorus.** Phosphorus was determined by the method of Meun and Smith [15].

**Carbohydrate analysis.** The proteins in polyacrylamide gels were stained for carbohydrate according to Kapitany and Zebrowski [16].

**Amino acid composition.** Amino acid analyses were carried out on a Beckman 119 CL amino acid analyzer using the standard protein hydrolyzate procedure. Samples were hydrolyzed at 110°C for 24 h with 5.7 M HCl containing phenol (10 μl/ml), in sealed evacuated tubes.

## Results and Discussion

Table I shows the distribution of solids and proteins from milks of control and colchicine-treated mammary glands. Although the volumes of

\* Reference to brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

TABLE I

RELATIVE AMOUNT OF SOLIDS, CASEINS, AND WHEY PROTEINS PRESENT IN MILKS OF CONTROL AND COLCHICINE-TREATED GOATS

Control volume was about 200 ml, while colchicine was about 100 ml.

		Total solids (g)	Recovered protein (g)		
			Casein	Whey	Whey ppt
Control	V18R	23	5.3	0.7	0.1
	Y90R	26	4.6	0.9	0.1
Colchicine	V18L	20	4.8	2.0	0.6
	Y90L	17	4.4	1.6	0.5

milk from colchicine-treated mammary udders were reduced 50% compared to the control, the relative amounts of caseins recovered are constant, while the amount of whey proteins from colchicine-treated udders are approx. 2-3-times that of the controls. The pH of 7.0 for colchicine-treated milk is significantly higher than pH 6.6 and 6.7 for controls; the higher pH and lower volumes in colchicine samples are consistent with the pH and volume changes accompanying alterations in mammary secretory capacity such as involution of the mammary gland [7,17].

Fig. 1. shows a typical disc-gel electrophoretic pattern (pH 8.6, 4 M urea) of control and colchicine casein and whey fractions. The colchicine sample shows a prominent band which moves with the marker dye in both whey and casein fractions, gels 2 and 4. It is designated III-protein. Another zone concentrated near the top of the lower gels, the I-protein, is very prominent in the colchicine casein fraction, gel 4. Both I- and III-fractions show mobilities similar to those found by Patton [6] for milk proteins of colchicine-treated goats. The colchicine whey and casein samples show more prominent minor bands than the controls, which might indicate proteolytic activity. Fig. 1 also shows two light bands designated II-fraction in both the casein and whey fractions of colchicine samples.

Gel electrophoresis in the presence of sodium dodecyl sulfate was carried out on these casein and whey samples (Fig. 2). Comparison of the casein samples (lanes 1 and 2) confirms the presence of the three novel bands produced in the casein fraction in response to colchicine infusion. In this case

the I-protein migrates slower than  $\beta$ -lactoglobulin; the III-protein, slightly faster than  $\beta$ -lactoglobulin, but slower than  $\alpha$ -lactalbumin;

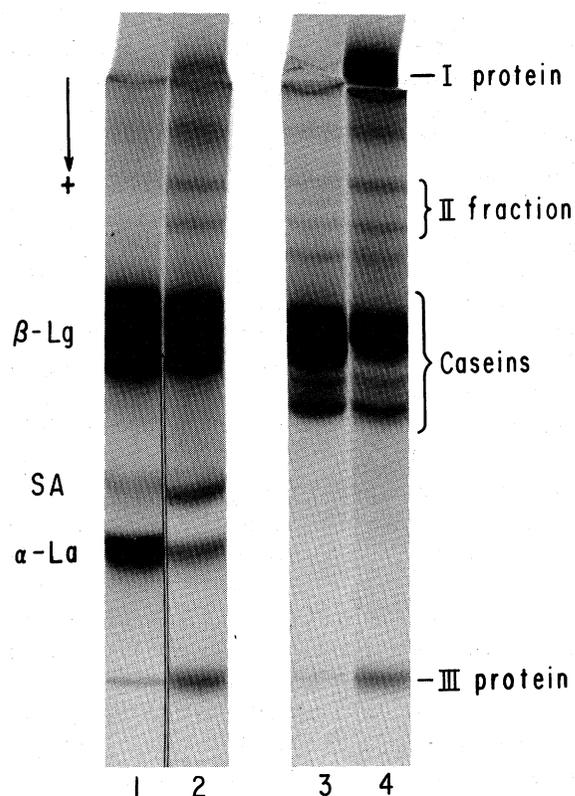


Fig. 1. Disc-gel electrophoretic pattern, pH 8.6, 4 M urea, of the whey and casein fractions from colchicine-treated and control mammary secretions: (1) control whey, (2) colchicine whey, (3) control casein, (4) colchicine casein. Abbreviations:  $\beta$ -Lg,  $\beta$ -lactoglobulin; SA, serum albumin;  $\alpha$ -La,  $\alpha$ -lactalbumin.

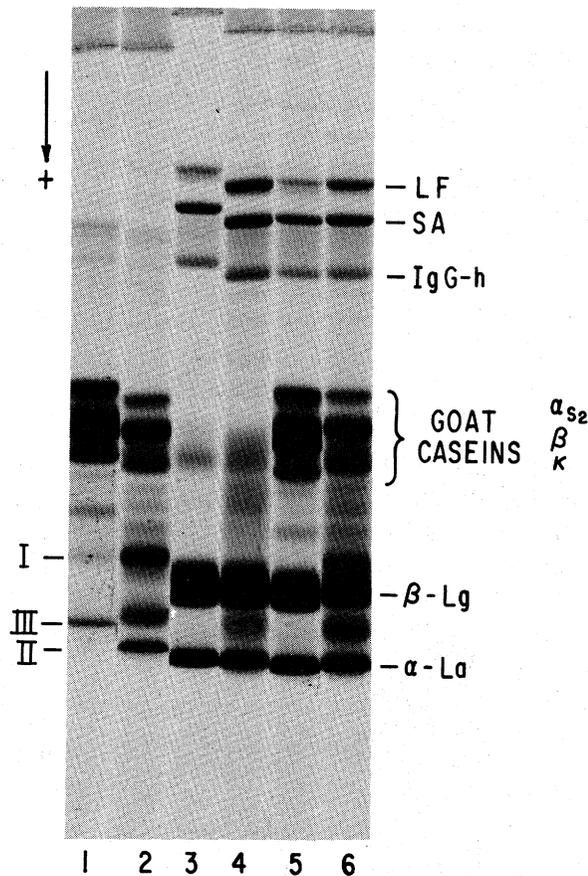


Fig. 2. Electrophoresis in the presence of SDS [10] of milk proteins from colchicine and control udders. Identification of bands was by comparison with known standards: (1) control casein, (2) colchicine casein, (3) control whey, (4) colchicine whey, (5) a 3/1 mixture of control casein and control whey, (6) a 3/1 mixture of colchicine casein and colchicine whey. Abbreviations: LF, lactoferrin; IgG-h, immunoglobulin G heavy chain.

migrates near the marker dye which moves with  $\alpha$ -lactalbumin in the Laemmli system. These bands are also present in the colchicine-treated whey (lanes 3 and 4), but the casein fractions were used to isolate the I-, II- and III-proteins.

Colchicine and control caseins were each chromatographed on DEAE-cellulose (Fig. 3). A major difference is seen in the relatively large first peak eluted from colchicine casein compared to the control. The column eluate was pooled as shown in Fig. 3, and proteins in the numbered fractions from colchicine casein were examined by

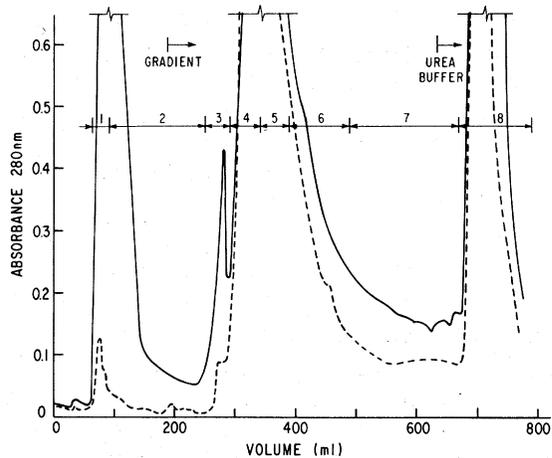


Fig. 3. 780 mg control (dashed line) and 740 mg colchicine casein (solid line) chromatographed on a DEAE-cellulose column. See Experimental section for details. Samples were pooled as indicated (fractions 1-8).

electrophoresis (Fig. 4). The first peak contains predominately the I-protein (Fig. 4, gels 1 and 2). A second difference in the elution profiles is observed for fraction 3 and shown in gel 3 of Fig. 4.

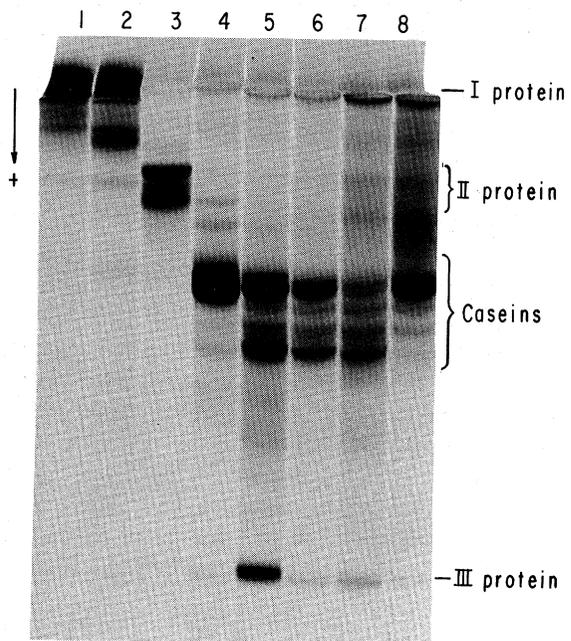


Fig. 4. Disc-gel electrophoresis, pH 8.6, 4 M urea, of colchicine casein eluted from DEAE-cellulose. In gel 5, much of the III-protein had been removed when this electrophoresis was run (see III-protein isolation in the text).

These two bands, designated II-fraction, are only faintly visible in the gels of the unfractionated control (Fig. 1). Finally, the fast-moving band, designated the III-protein, is found in fraction 5, Fig. 3, and gel 5 of Fig. 4. The gels in Fig. 4 numbered 1-2, 3, and 5 contain the crude I-, II- and III-fractions, respectively (compare with Fig. 1, lanes 3 and 4). The crude fractions 1-2 (I-protein) and 3 (II-fraction) were each rechromatographed on Sephadex G-50 (see Experimental). From the I-protein, two fractions designated I-1 and I-2 were obtained by further chromatography on CM-cellulose. Fig. 5 shows disc-gel electrophoretic patterns of I-1, I-2 and II-proteins at acid and alkaline pH. The I-1 protein has two minor bands as impurities compared to I-2 at alkaline pH, while at acid pH I-2 shows a doublet resembling genetic polymorphism observed for some milk proteins. The major contaminants of I-1 and I-2 which were seen in Fig. 4, lanes 1 and 2, are thus removed, and both fractions gave essentially one equivalent band on SDS-gel electrophoresis. In the case of the II-fraction, the Sephadex G-50 treatment removed the slower moving component (Fig. 4, lane 3) and yielded a component with essentially one band in both acid and alkaline urea

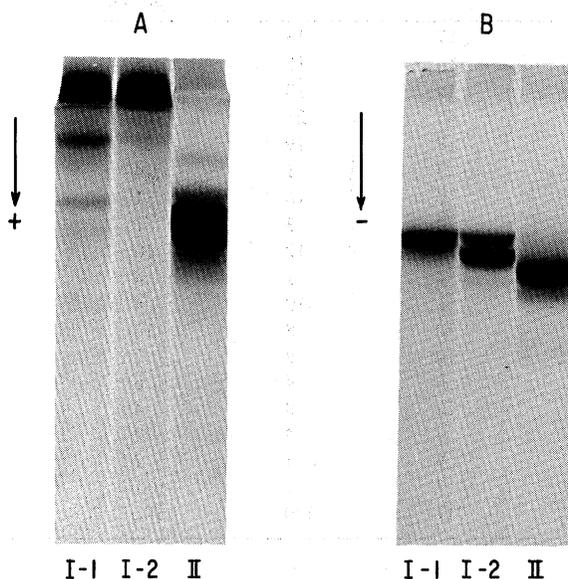


Fig. 5. Disc-gel electrophoresis (a) pH 8.6, 4 M urea, and (b) pH 4.3, 8 M urea, of I-1, I-2 and II-proteins.

electrophoresis (Fig. 5A and B) and SDS-gel electrophoresis. These samples were used for amino acid analysis.

Fig. 6 shows the gel filtration profile of III-fraction on Bio-Gel-P 100. The casein contaminants (Fig. 4, lane 5) were removed by acid precipitation (see Experimental). The disc-gel electrophoretic pattern at alkaline pH, 4 M urea, shows some diffuse slower-moving carbohydrate-positive material for III-1. On molecular weight determinations using SDS, the gels (not shown) of III-2 and III-3 have single bands, while III-1 has, besides the major band corresponding to III-2 and III-3, two minor bands of slower mobility. The III-protein is quite acidic; with polyacrylamide gel electrophoresis at pH 4.3, 8 M urea, it moves from the gels into the upper buffer. When slab gels, not shown, were run at a low pH of 3.0, two bands of equal intensity were observed which moved into the gels about 1.1 and 1.5 cm. The III-protein appears to occur in different states of aggregation, as indicated by the number of peaks containing III-protein, eluted from the gel filtration column (Fig. 6). Rechromatography of this protein yielded

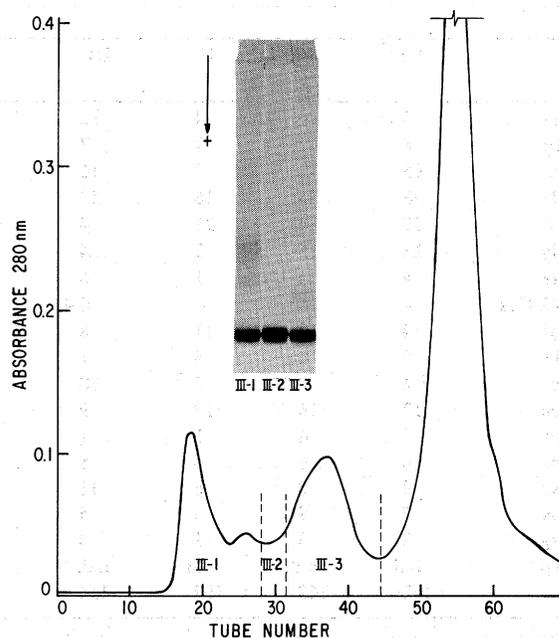


Fig. 6. Gel filtration of III-fraction on Bio-Gel P 100. Inset is disc gel pattern, pH 8.6, 4 M urea, of isolated fractions III-1, III-2 and III-3.

similar results, with little or no rearrangement of peaks in the absence of dissociating and reducing agents.

The molecular weight following the method of Weber and Osborn [11] for I-, II-, and III-proteins are 19400, 12000 and 18200, respectively. These are average values of several runs. Slight impurities of 16000 and 17000 molecular weight proteins were observed in I-proteins at higher sample loadings.

The III-protein contains 1.6% phosphorus, while no phosphorus was found in the I- and II-proteins, and they are not positive for carbohydrate.

The amino acid composition of I-, II- and III-proteins together with the normal goat milk caseins and a proline-rich peptide from sheep colostrum are shown in Table II. Since the I-, II- and III-proteins contain no cystine, the goat whey proteins are not included in this comparison. Variations between I-1 and I-2 proteins are probably a reflection of minor impurities.

Janusz et al. [22] isolated and characterized the proline-rich polypeptide from sheep colostrum. It showed a minimum molecular weight of 6000 by gel filtration in guanidine-HCl; polyacrylamide electrophoresis in the presence of SDS revealed a major band of 6000 and minor bands of 12000 and 18000 molecular weights. The two higher molecular weight bands were described as nondissociated aggregates of the 6000 molecular weight polypeptide.

As evidenced by polyacrylamide gel electrophoresis, III-protein is a very acidic protein in which, besides the phosphate groups, glutamic acid or glutamine residues comprise about one-third of the molecule (Table II). In contrast to the III-protein, but like  $\beta$ -casein, I-protein has a high proline content. When the molecular weights and nearest integer values are considered the III-protein and also the I-proteins cannot be fragments of the  $\beta$ -,  $\alpha_s$ - and  $\kappa$ -caseins of goat milk. The II-protein and sheep proline-rich peptide appear to be related.

TABLE II

COMPARISON OF AMINO ACID COMPOSITION (RESIDUES/MOLECULE) OF I-, II- AND III-PROTEINS WITH GOAT CASEINS AND SHEEP COLOSTRUM PROLINE-RICH PROTEIN

	I-1	I-2	II	III	$\beta_{1,2}$ -Cas <sup>a</sup>	$\alpha_s$ -Cas <sup>b</sup>	$\kappa$ -Cas <sup>c</sup>	Sheep protein <sup>d</sup>
Asx	5	6	4	12	9	17	15	4
Thr	8	7	7	7	12	14	15	8
Ser	15	17	7	16	15	14	13	6
Glx	23	23	16	52	43	45	26	12
Pro	27	24	21	4	33	18	19	22
Gly	13	15	2	4	6	4	1	4
Ala	4	4	1	6	5	10	16	1
1/2 Cys	—	—	—	0.4	—	2	3	1
Val	14	13	11	8	21	12	12	10
Met	5	4	4	1	6	4	1	4
Ile	4	4	2	10	9	11	10	4
Leu	17	16	12	9	20	12	8	12
Tyr	3	4	1	1	4	11	9	2
Phe	6	6	4	5	9	8	4	6
Lys	8	8	6	11	12	22	8	4
His	7	8	3	5	5	5	4	2
Arg	2	3	1	3	3	6	5	2.5
Trp	n.d.	n.d.	n.d.	n.d.	1	2	1	n.d.
	161	162	102	154	213	217	170	102

<sup>a</sup> Richardson and Creamer [18].

<sup>b</sup> Richardson and Creamer [19].

<sup>c</sup> Addeo et al. [20].

<sup>d</sup> Janusz et al. [22].

They have the same molecular weight based on their amino acid composition and using the Cornish-Bowden [21] calculation relating two proteins of the same size by amino acid composition, an  $S\Delta n$  value of 0.1 N is obtained. If the  $S\Delta n$  is less than 0.42 N [21], 'there is a strong indication amounting almost to certainty' that the II- and sheep proline-rich proteins are related to each other. They are not related to other types of proline-rich proteins [23].

Studies on a possible biological role of the sheep colostrum proline-rich protein showed that it increases the permeability of skin and that it has a regulatory activity, stimulating the T-cells of the immune system [22,24]. Whether the II-protein would exhibit similar properties, and how this would relate to the effects of colchicine on the production of milk proteins, are interesting questions, as is the biological origin of the protein.

Based upon the analysis of amino acid profiles and phosphorus content, the very acidic III-protein is a phosphoprotein unlike any goat casein. If the I-, II- and perhaps III-proteins are produced in response to an effect of colchicine on the cell microtubules, and not by proteolysis of the caseins, it is difficult to explain their presence in milk. The III-protein does not appear to be related to the calcium-binding protein superfamily, muscle parvalbumin and troponin [25]. However, highly acidic proteins have been isolated from brain tissue [26]. The production of the acidic III-protein may in some unknown way be initiated in response to an effect of colchicine on the nervous system of the mammary gland. It is unlikely that the secretory cells are permanently damaged by colchicine, because the system is reversible, and on omitting the colchicine treatment the milk production of the mammary gland apparently becomes normal in time [5,27].

A complicating factor in colchicine treatment of mammary gland is that Oliver and Smith [7] found that colchicine treatment caused a visible inflammation in cow udders, accompanied by increased serum albumin content, while Darton and McDowell [28] demonstrated increased IgG transport as a result of colchicine treatment. If infusion of colchicine in goat mammary gland leads to inflammation as suggested by Oliver and Smith for cow, an increase in lactoferrin might be expected,

since lactoferrin is released from neutrophils at the site of infection during an inflammatory period [29]. Some major protein differences were observed in the high molecular weight range for colchicine whey vs. control whey (Fig. 2). Gels 3 and 4 show a small increase of serum albumin and heavy chain IgG, but a dramatic increase in lactoferrin content. Antiserum to bovine lactoferrin showed a 10–15-fold increase in lactoferrin content of the whey protein fraction obtained from colchicine-treated goat mammary gland. The lactoferrin content of milk may also rise as the normal secretory protein content drops as in the case of involution [17]. This could argue for paracellular transport of these novel proteins.

In this study it was found that the amounts of casein produced by the control and colchicine-treated udder are about the same, but the amount of protein in whey produced by colchicine is significantly higher (Table I). This finding suggests that regulation of the production of caseins differs from that of the whey proteins. In summary, colchicine infusion into goat mammary gland produced quantitative changes in milk proteins, as well as the occurrence of three novel groups of proteins. The I- and II-proteins are proline-rich, the latter is compositionally related to a cell surface active proline-rich protein from sheep colostrum. The III-protein is a very acidic phosphoprotein.

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