J. Physiol. (1981) **313**, *pp.* 561–570
With 5 text-figures
Printed in Great Britain

IONIZED CALCIUM IN MILK AND THE INTEGRITY OF THE MAMMARY EPITHELIUM IN THE GOAT

BY M. C. NEVILLE* AND M. PEAKER†

From the Agricultural Research Council Institute of Animal Physiology, Babraham, Cambridge CB2 4AT, and the Hannah Research Institute, Ayr KA6 5HL

(*Received 24 September 1980*)

SUMMARY

1. Injection of citrate or EGTA solutions into the lumen of the mammary gland of goats in quantities sufficient to reduce ionized calcium to less than one-tenth of normal, led to increases in milk concentrations of Na and Cl and decreases in K and lactose.

2. Subsequent milk yields were decreased in glands treated with citrate but not in those treated with EGTA.

3. Blood–milk potential difference decreased (i.e. towards zero) in glands in which citrate was present.

4. In goats milked hourly with the aid of oxytocin, milk Na and Cl concentrations increased while K and lactose decreased; there was no apparent decrease in Ca^{2+} concentration.

5. It is suggested that ionized calcium in milk is essential to preserve the integrity of the mammary epithelium during lactation.

INTRODUCTION

When tissues are exposed to low-calcium media, the integrity of the *zonulae occludentes* between cells is not maintained (see, for example, Amsterdam & Jamieson, 1974; Galli, Brenna, Camilli & Meldolesi, 1976). In confluent cultures of mid-pregnant mouse mammary gland, Pitelka & Hamamoto (1977 and personal communcation) used morphological techniques to show that exposure to calcium-free media containing EGTA caused the appearance of discontinuities in the occluding zones within 5 min. Other changes included cytoplasmic contraction beneath junctional belts with development of long interdigitating filopodia and gradual endocytosis of junction patches. In these studies, calcium was removed, of necessity, from both baso-lateral and apical surfaces of the mammary epithelium.

The partition of calcium in milk is complex: some calcium is protein-associated (about 70% in the goat, Neville & Peaker, 1979) while the majority of that in the soluble fraction occurs as the monovalent anion, calciocitrate (see Holt, Muir, Ormrod, Zammit & Peaker, 1980). Nevertheless, chemical and electrochemical determinations

* Permanent address: Department of Physiology, University of Colorado Medical Center, Denver, Colorado 80220, U.S.A.

† to whom requests for reprints should be addressed.

0022-3751/81/4580-1231 $07.50

indicate that there is some free Ca^{2+} in milk (see Jenness, 1974; Baumrucker, 1978) and, in the goat, determinations using a calcium electrode give a value of approximately 1 mM in a total calcium concentration of 27 mM (see Neville & Peaker, 1979).

The question therefore arises of whether the ionized calcium in milk is important for the maintenance of the integrity of the mammary epithelium. Since integrity of the occluding junctions is vital for milk of normal composition to be produced (see Peaker, 1978) we have investigated whether chelation of the Ca^{2+} in milk has effects on milk composition and on transepithelial potential difference (p.d.) which are compatible with an effect on junctional integrity. To achieve chelation, we have added either additional citrate or ethyleneglycol-bis (β-amino-ethyl ether) *N,N'*-tetra-acetic acid (EGTA) to milk within the mammary gland of goats.

METHODS

Animals. Experiments were conducted on lactating goats in which a mammary ('milk' or caudal superficial epigastric) vein was exteriorized in a loop of skin (Linzell, 1960). The goats were milked at approximately 09.00 and 16.00 hr each day; the time of milking and the yield of each gland were recorded.

Conduct of experiments. For studies on milk yield and composition, sterile solutions of sodium citrate (50 mM, $Na_3C_6H_5O_7 . 5H_2O$) in sucrose (240 mM) and of 300 mM-Na-EGTA (pH 6·8) were prepared and Millipore-filtered immediately before use. In three goats, 250 ml. of the citrate–sucrose solution was injected aseptically into the lumen of one mammary gland (via the teat canal) approximately 3 hr after morning milking. In two goats, 26 and 67 ml. of the EGTA was injected. Three hours later both glands were milked; normal twice-daily milking was then continued.

For studies on blood-milk p.d. (determined as described by Peaker, 1977), goats were milked approximately 4 hr after morning milking; the initial p.d. was recorded and 250 ml. of the citrate–sucrose solution injected.

For studies on the effect of frequent milking on milk composition, goats were milked hourly for 6 hr, starting at morning milking; 100 mu. oxytocin was injected I.V. each time (Linzell & Peaker, 1971).

Analytical methods. For the citrate and frequent milking experiments, milk Na, K, Cl and lactose were determined as described by Fleet, Linzell & Peaker (1972). For the EGTA experiments similar methods for Na, K and Cl were adapted to a Technicon auto-analyzer II; lactose (as total reducing sugar by an alkaline ferricyanide method) was also determined in the auto-analyzer as described in Technicon industrial method no. 120–71A. Milk Ca^{2+} was estimated using a Radiometer (Copenhagen) electrode (model F2112Ca) (where possible allowing for total ionic strength of standards and milks), and electrical conductivity as described by Linzell, Peaker & Rowell (1974).

RESULTS

Milk yield and composition

Effects of citrate

The changes induced by the sucrose–citrate solution in three goats were similar. Therefore, data have been pooled in Figs. 1 and 2 as means $\pm$S.E. of means.

In Fig. 1 are plotted the mean milk yields of the treated glands as percentages of the yields of the untreated glands. For the afternoon milk yield on the day of the experiment, the value shown is the combined yield from the milking when the citrate–sucrose solution was removed and the normal afternoon milking minus the volume of solution injected. There was a tendency for yield to be increased during and immediately after the experiment. In the 2 days following treatment, the yield

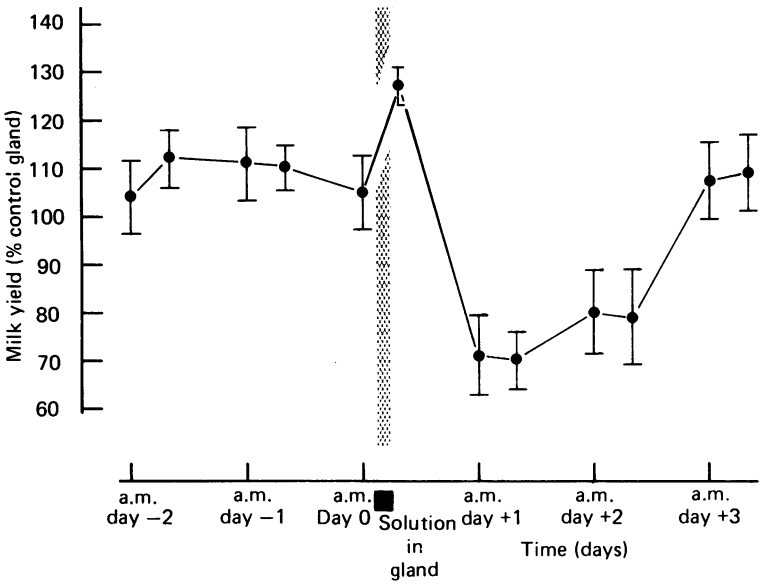

Fig. 1. Effect on milk yield of introducing 250 ml. citrate–sucrose solution into one mammary gland of each of three goats (see text for details). Mean ± S.E. The first point shown after treatment is the combined yield from the milking when the solution was removed and that of the normal afternoon milking.

of the treated glands appeared to be decreased, the main fall occurring in the period from 2–18 hr after removal of the solution.

At the milking when the citrate–sucrose solution was removed, milk Na concentration was increased while K, Cl and lactose were decreased (Fig. 2). However, it is difficult to interpret changes at this milking because of four complicating factors: (i) the presence of the additional volume of liquid in the gland would tend to depress concentrations by a dilution effect; (ii) the presence of additional Na in the citrate–sucrose solution, although when this is allowed for the Na concentration was still increased; (iii) the tendency for Na, K and Cl to pass into the diluted milk from the cell as a result of altering the concentration gradients (Linzell, Mepham & Peaker, 1976); (iv) the additional citrate could affect anion movements by a Donnan or other effect. Therefore, interpretation of the effects of the solution is not based on changes at this milking.

At the milking approximately 2 hr after the solution was removed, the milk had a markedly different composition from that obtaining before treatment and from that of the untreated gland (Fig. 2). Mean milk Na and Cl concentration were approximately 40 and 20 m-mole/l. respectively higher than in the untreated gland, while K and lactose were approximately 20 and 57 m-mole/l. respectively lower. Therefore, it appears that treatment with the citrate–sucrose solution increased milk Na and Cl and decreased K and lactose concentrations (even with the small number of animals, these differences were significant ($P < 0{\cdot}05$) with respect to the untreated gland). This tendency was still apparent at the milking 18 hr after removal of the solution.

It is pertinent to consider the immediate effect of adding 250 ml. citrate–sucrose solution on the ionized Ca^{2+} of the milk within the gland. The effect is not simply one of chelating the Ca^{2+} in milk. Holt & Muir (1979) have shown that when citrate

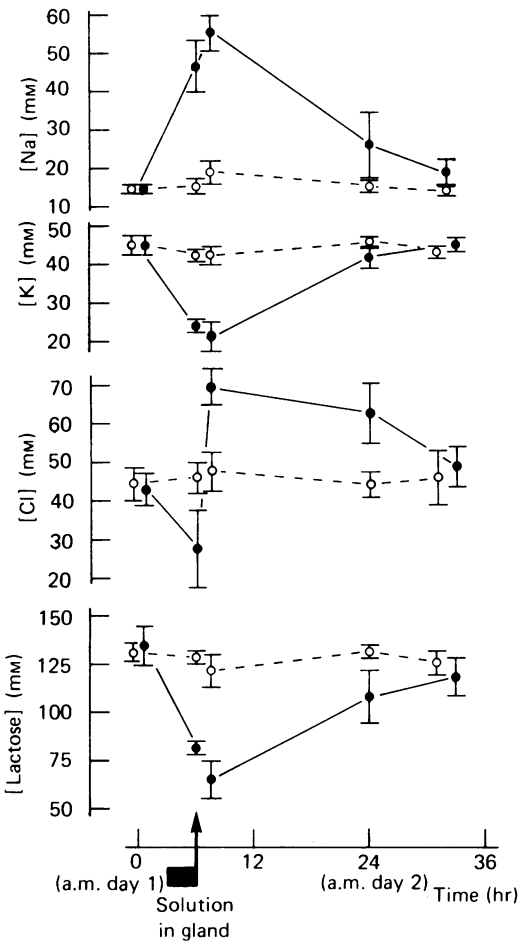

Fig. 2. Effects on milk composition of introducing 250 ml. citrate–sucrose solution into a mammary gland of three goats (see text for details). ●——●, treated glands; ○---○, untreated glands. Mean ± S.E.

is added to milk *in vitro*, the proportion of 'soluble' calcium increases, indicating that the calcium being chelated is drawn from the protein-associated pool to a great extent; they also showed that Ca^{2+} concentration is not affected by small additions of citrate. We have therefore added larger amounts of citrate to milk *in vitro* to determine the likely concentration of Ca^{2+} in 'milk' after addition of 250 ml. of the citrate–sucrose to the 210 ml. of milk we calculated from previous milk yields to be in the gland. It can be seen from Fig. 3 that this addition would have reduced the concentration of Ca^{2+} by a factor of approximately 12 to about 0·1 mM.

At the end of the 3 hr period with the solution in the gland, the mean volume of milk as a percentage of the total liquid in the gland was 59 ± 2·8 (S.E.). If no additional movement of calcium occurred into milk during this period, the likely Ca^{2+} concentration within the lumen would have been approximately 0·45 mM (Fig. 3).

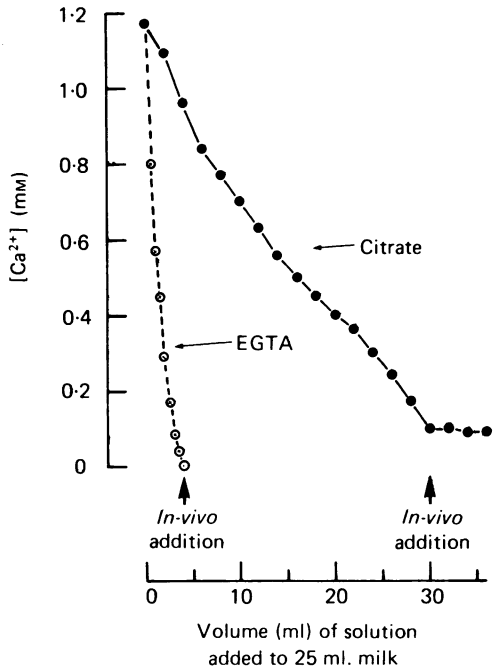

Fig. 3. Effects of adding citrate–sucrose solution (50 mM; 240 mM-sucrose) or 300 mM-Na-EGTA to milk *in vitro* on Ca^{2+} at 25 °C. The calcium electrode was calibrated against $CaCl_2$ solutions of similar ionic composition to normal goat's milk, and showed a 37 mV change per tenfold change in Ca^{2+} concentration (expressed as mM). The arrows show the approximate dilutions used for the experiments *in vivo*.

Effects of EGTA

In the two goats given EGTA, it was calculated from the previous rates of milk secretion (see below), as well as the amounts of 300 mM-EGTA given (26 and 67 ml.) and the curve showing the effects of adding the EGTA solution to milk *in vitro* (Fig. 3), that the immediate effect on Ca^{2+} concentration in the lumen would have been a reduction to $< 0{\cdot}01$ mM.

The effects on milk composition, again judged from the milking after the solution was removed, were qualitatively similar in both goats to those achieved with citrate, i.e. Na and Cl concentrations increased while K and lactose decreased; the results for one goat are shown in Fig. 4. However, in contrast to the results obtained with citrate, in neither goat was the milk yield of the treated gland apparently decreased after treatment, the yields being 54 and 140 ml./hr respectively before treatment and 55 and 140 ml./hr in the 2 days after treatment.

In these studies, additional small milk samples were taken immediately before treatment and Ca^{2+} concentrations estimated in these and in the milk obtained when the solution was removed. In view of the difficulties involved in calibrating a calcium electrode in conditions when ionic strength has changed, the results in Table 1 are presented simply as mV readings. It can be seen that Ca^{2+} concentration was still

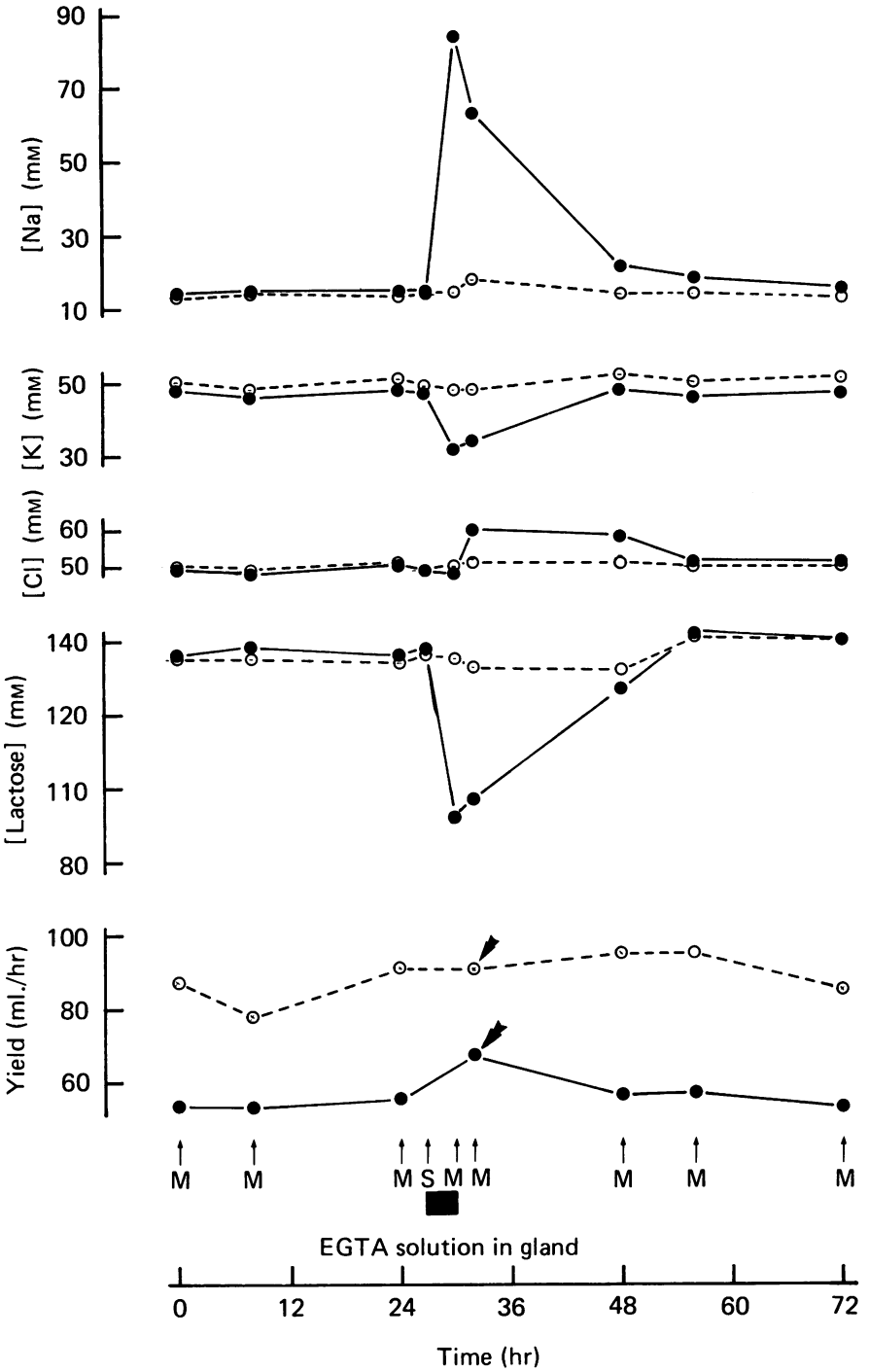

Fig. 4. Effect of introducing 36 ml. 300 mM-EGTA into the lumen of one mammary gland of a goat on milk yield and composition. ●——●, treated gland; ○---○, untreated gland. The points marked by double arrows are the combined yields from the milking when the solution was removed, and those of the normal afternoon milking. Single arrows show times of milking (M) or taking of 10 ml. milk samples (S).

TABLE 1. Effect of 300 mM-EGTA introduced into the mammary lumen (i) on milk [Ca^{2+}] 3 hr later and at the milking following removal of the solution (see Fig. 4) in two goats, and (ii) on mammary venous plasma 2·5 hr later. The results are expressed as mV (see text) and estimations were made at 25 °C. The electrode used showed a 25 mV change per tenfold change in concentration of Ca^{2+}. A value of 40 mV was approximately equivalent to a concentration of 1·6 mM-Ca^{2+}

	Milk			
	Goat 1		Goat 2	
	Treated	Untreated	Treated	Untreated
Before	39	39	40	40
After 3 hr	27	39	19	39
Next milking	38	39	40	40

	Mammary venous plasma	
	Goat 1	Goat 2
Before	21	24
After 2·5 hr	25	27

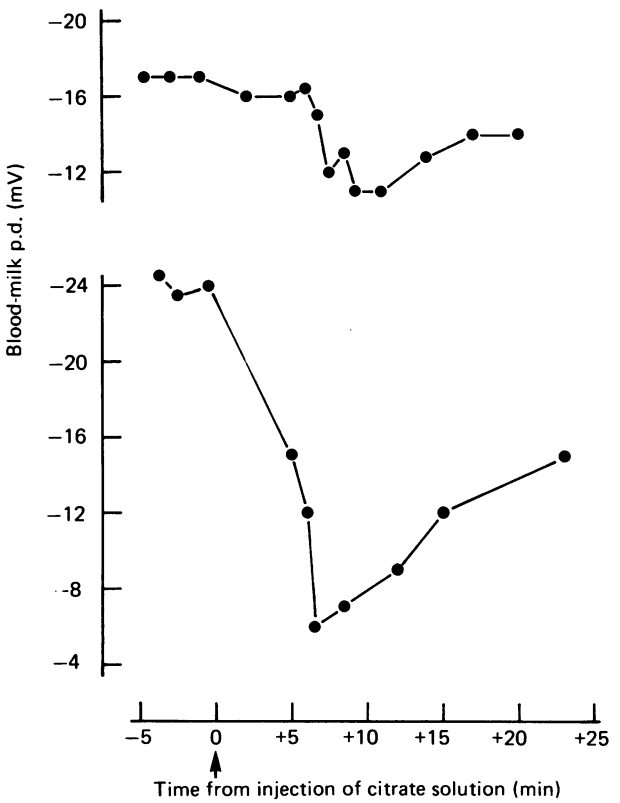

Fig. 5. Effects of introducing 250 ml. 50 mM-citrate/240 mM-sucrose solution into the lumen of a mammary gland in two goats on blood–milk p.d. (blood = 0).

apparently decreased at the end of the 3 hr period but was restored to pre-treatment values at the milking approximately 2 hr later. Also shown in Table 1 are Ca^{2+} estimations from mammary venous plasma taken immediately before and 2·5 hr after the solution was injected; it is clear that there was no apparent decrease in the concentration of Ca^{2+}.

Blood–milk p.d.

In the two goats treated with the citrate–sucrose solution, the initial blood–milk p.d. was −17 and −24 mV (milk negative) respectively. After treatment p.d. decreased (i.e. milk became less negative). The maximum fall over the 20–23 min recordings were made was 6 mV in one goat and 18 mv in the other (Fig. 5).

Changes in milk composition during hourly milking

During hourly milking with the aid of oxytocin (100 mu. each time), we found previously that milk Na and Cl concentrations increased while K and lactose decreased (Linzell & Peaker, 1971). The changes were interpreted as due to a decrease in junctional integrity. To determine whether this was due to an alteration in the milk ionized Ca^{2+}, we milked goats at hourly intervals for 6 hr, injecting 100 mu. oxytocin I.V. at each time. The Na and Ca^{2+} concentrations were determined on each sample. While the mean maximum increase in Na was 16 m-mole/l. at the fourth hour (an approximate doubling) there was no apparent change in Ca^{2+} apart from a tendency for a slight increase at the first hour. Taking all the data from the three goats for the first to sixth hour there was no significant correlation between the change in Na concentration and that of Ca^{2+} ($r = 0{\cdot}012$).

In studies of this type with Ca^{2+} determinations made on fresh milk with a calcium electrode it is difficult to compensate for changes in ionic strength at the time of measurement. However, as judged from electrical conductivity measurements, the maximum rise was equivalent to a mean rise of 6 m-mole/l. of a NaCl solution. Moreover, there was no correlation, using the same samples as above, between conductivity and Ca^{2+} concentration ($r = 0{\cdot}276$).

DISCUSSION

The results strongly indicate that the presence of citrate–sucrose or EGTA solutions in the mammary gland disrupts the integrity of the epithelium. In all other circumstances in which milk Na and Cl concentrations are increased while K and lactose are decreased there is evidence from for example, movements of isotopically labelled disaccharides, that the junctions between cells are leaky (see Peaker, 1978). Moreover, the decrease in transepithelial (blood–milk) p.d. is also compatible with the view that the two sides of the epithelium are partially short-circuited in the presence of citrate or of EGTA (Peaker, 1977, 1980).

While Pitelka & Hamamoto (1977) have shown *in vitro* that chelation of Ca^{2+} leads to disruption of the occluding junctions, the present results indicate that, *in vivo*, Ca^{2+} is required on the milk (luminal) side to maintain epithelial integrity. Whether the decrease in milk yield after treatment with citrate–sucrose is related to long-term effects of chelation of Ca^{2+} is open to question since this effect was not observed with EGTA. The effect of treatment on intracellular calcium was not assessed.

It is not possible to assess, from the present studies, the magnitude of the decrease

in milk Ca^{2+} concentration required for epithelial integrity to break down. While substantial dilution of milk *in vivo* with isosmotic sucrose or lactose solutions does not lead to the changes observed with citrate included (Linzell *et al.* 1976; Peaker, 1977; Neville & Peaker, 1979), it seems likely that Ca^{2+} could be replenished by re-equilibration of reactions between Ca^{2+}, calciocitrate and protein-associated calcium under these circumstances.

If Ca^{2+} in milk is important for epithelial integrity, the question arises of whether calcium movements or binding control milk composition when the proposed epithelial paracellular pathway through leaky junctions (see Linzell & Peaker, 1971; Peaker, 1978) opens or closes. While the present results indicate that this is not a likely means of control in the effect of frequent milking on epithelial permeability and milk composition, the possibility of control by this means at such times as the onset and cessation of lactation cannot be ruled out; experiments are in progress to investigate the Ca^{2+} concentrations in mammary secretions at these times.

There is circumstantial evidence that changes in the concentration of Ca^{2+} on the blood or milk side might be important in altering junctional permeability in pathological conditions. Linzell & Peaker (1972) noted that milk Na and Cl concentrations increased and K and lactose decreased in a lactating cow suffering from hypocalcaemia, and a similar effect has been observed in a case of hypocalcaemia several days after parturition (M. Peaker & F. M. Maule Walker, unpublished).

Thus while the ionized Ca^{2+} is a small proportion of the total calcium content of milk and is of negligible nutritional importance, it appears to have a major biological role in preserving the integrity of the mammary epithelium. In a wider context, it may be pertinent to consider whether Ca^{2+} in other exocrine secretions plays a similar role.

We are most grateful to Mr I. R. Fleet, Mrs J. A. Goode and Mr R. Proudfoot (Institute of Animal Physiology) and Mr D. R. Blatchford, Dr P. L. Clarke, Mr T. F. Hutchinson and Mr R. M. Mabon (Hannah Research Institute) for assistance and care of the animals.

REFERENCES

AMSTERDAM, A. & JAMIESON, J. D. (1974). Studies on dispersed pancreatic exocrine cells. I. Dissociation technique and morphologic characteristics of separated cells. *J. cell Biol.* **63**, 1037–1056.

BAUMRUCKER, C. R. (1978). Calcium transport in lactation. In *Lactation*, vol. 4, ed., LARSON, B. L., pp. 463–474. New York: Academic.

FLEET, I. R., LINZELL, J. L. & PEAKER, M. (1972). The use of an autoanalyzer for the rapid analysis of milk constituents affected by subclinical mastitis. *Br. vet. J.* **128**, 297–300.

GALLI, P., BRENNA, A., CAMILLI, P. D. E. & MELDOLESI, J. (1976). Extracellular calcium and the organization of tight junctions in pancreatic acinar cells. *Expl Cell Res.* **99**, 178–183.

HOLT, C. & MUIR, D. D. (1979). Inorganic constituents of milk. I. Correlation of soluble calcium with citrate in bovine milk. *J. Dairy Res.* **46**, 433–439.

HOLT, C., MUIR, D. D., ORMROD, I. H. L., ZAMMIT, V. A. & PEAKER, M. (1980). Citrate in Milk. *Hannah Research Institute, Report 1979*, pp. 63–69. Ayr: Hannah Research Institute.

JENNESS, R. (1974). The composition of milk. In *Lactation*, vol. 3, ed. LARSON, B. L. & SMITH, V. R., pp. 3–107. New York: Academic.

LINZELL, J. L. (1960). Mammary-gland blood flow and oxygen, glucose and volatile fatty acid uptake in the conscious goat. *J. Physiol.* **153**, 492–509.

LINZELL, J. L., MEPHAM, T. B. & PEAKER, M. (1976). The secretion of citrate into milk. *J. Physiol.* **260**, 739–750.

LINZELL, J. L. & PEAKER, M. (1971). The effects of oxytocin and milk removal on milk secretion in the goat. *J. Physiol.* **216**, 710–716.

LINZELL, J. L. & PEAKER, M. (1972). Day-to-day variations in milk composition in the goat and cow as a guide to the detection of subclinical mastitis. *Br. vet. J.* **128**, 284–296.

LINZELL, J. L., PEAKER, M. & ROWELL, J. G. (1974). Electrical conductivity of foremilk for detecting subclinical mastitis in cows. *J. agric. Sci., Camb.* **83**, 309–325.

NEVILLE, M. C. & PEAKER, M. (1979). The secretion of calcium and phosphorus into milk. *J. Physiol.* **290**, 59–67.

PEAKER, M. (1977). Mechanism of milk secretion: milk composition in relation to potential difference across the mammary epithelium. *J. Physiol.* **270**, 489–505.

PEAKER, M. (1978). Ion and water transport in the mammary gland. In *Lactation*, vol. 4, ed. LARSON, B. L., pp. 437–462. New York: Academic.

PEAKER, M. (1980). The effect of raised intramammary pressure on mammary function in the goat in relation to the cessation of lactation. *J. Physiol.* **301**, 415–428.

PITELKA, D. R. & HAMAMOTO, S. T. (1977). Calcium-chelation-induced disruption of occluding junctions by cultured mammary epithelial cells. *J. cell Biol.* **75**, 69a.