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THE SECRETION OF CITRATE INTO MILK

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SUMMARY

1. The time course of changes in specific activities of citrate, lactose and fatty acids in milk during frequent milking, following the I.V. administration of labelled glucose, acetate and chylomicrons in goats has been studied. Peak specific activities of lactose and citrate in milk were reached at 2–3 hr, while peak specific activities of fatty acids were reached at 5–7 hr.

2. Following short I.A. infusions of ^{24}Na, ^{36}Cl and ^{42}K, peak specific activities in milk were reached in 1 hr or less.

3. The mammary epithelium of lactating goats was found to be virtually impermeable to labelled citrate in both directions.

4. Labelled citrate had an apparent volume of distribution in lactating guinea-pigs mammary slices *in vitro* similar to that of extracellular space markers.

5. Treatment of goats with large doses of oxytocin markedly increased the permeability of the secretory epithelium to labelled citrate.

6. In the goat mammary gland, citrate, protein and calcium failed to enter milk which had been diluted with isosmotic lactose by intraductal injection, whereas Na, K and Cl did enter, thus tending to restore the concentrations of these ions to normal.

7. It is suggested that citrate, which is formed within the secretory cell, enters milk not by passage across the apical cell membrane but, in common with lactose and milk protein, by exocytosis of Golgi vesicles. It appears that citrate is held at high concentrations in milk by virtue of the impermeability of the mammary epithelium to the forms in which it occurs in milk.

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INTRODUCTION

Citrate is present in the milk of many animals in significant amounts. In cows and goats the concentration is approximately 150 mg/100 ml. (Davies & White, 1960; Fleet, Goode, Hamon, Laurie, Linzell & Peaker, 1975) and in human milk approximately 80 mg/100 ml. (see Macy, Kelly & Sloan, 1953).

Although citrate plays a central role in metabolism, the significance of its presence in milk is a matter of speculation and its mode of secretion is unknown. Therefore we have attempted to elucidate the mode of secretion and the mechanism by which it is held at high concentrations during storage in the alveoli and ducts of the mammary gland.

Citrate forms one of the main buffer systems of milk (see Davies, 1939) and at the pH of milk (6·3–6·4 in goats, Linzell & Peaker, 1975) it is anticipated that approximately half would be present as $HCit^{2-}$ and half as Cit^{3-}, the pK_a of this dissociation being 6·41 (throughout we shall abbreviate the citrate moiety as Cit). However, Cit^{3-} chelates calcium and magnesium to form soluble complexes which appear to act as monovalent anions (calciocitrate and magnesiocitrate, $CaCit^-$ and $MgCit^-$) (Hastings, McLean, Eichelberger, Hall & DaCosta, 1934) and since calcium is present in excess, it is unlikely that Cit^{3-} is present in milk in the free form. Moreover approximately 7% of the total citrate in cows' milk is in the disperse, i.e. non-aqueous phase, probably combined in calcium–casein complexes (Davies & White, 1960).

Some of the data on the time course of citrate secretion from labelled precursors have been presented as a preliminary communication to the Biochemical Society (Hardwick, Linzell & Mepham, 1963*a*).

METHODS

Animals. Experiments were conducted on lactating goats in which a mammary ('milk' or caudal superficial epigastric) vein and a carotid artery were exteriorized in loops of skin (Linzell, 1960, 1963*a*). Some of the animals also had one mammary gland transplanted to the neck with the artery and vein anastomosed to an exteriorized carotid artery and jugular vein respectively (Linzell, 1963*b*); this enabled catheterization and close-arterial infusion to be carried out without further surgery by Seldinger's (1953) technique under local anaesthesia.

For some experiments mammary tissue was obtained from guinea-pigs lactating for 3–5 days.

Time course of secretion of citrate and other milk constituents. After morning milking of two goats, two doses of oxytocin (200 mu. each time, I.V.) were given, and the animals again milked after each dose; this was done to remove as much as possible of the milk formed before the experiment. In one goat 23 μc [2-^{14}C]acetate and 13·5 μc ^{3}H-labelled chylomicrons ([^{3}H]stearate incorporated into triglycerides), prepared as described by Lascelles, Hardwick, Linzell & Mepham (1964), were

given as a single I.V. injection. The other goat received 23 μc [U-^{14}C]glucose and 13·5 μc ^{3}H-labelled chylomicrons. Thereafter the goats were milked every hour for 8 hr following the I.V. injection of 200 mu. oxytocin each time. The specific activities (S.A.) of citrate, lactose and fatty acids were determined using the methods described by Hardwick, Linzell & Mepham (1963*b*).

In other experiments on two goats, ^{24}Na, ^{36}Cl and ^{42}K were infused I.A. for 14 min on separate occasions, 2 hr after the start of hourly milking (100 mu. oxytocin I.V. each time). The animals were milked within 25 min of the end of the infusion and then at intervals of approximately 1 hr. Specific activities were determined as described by Linzell & Peaker (1971 *c*).

Permeability to citrate. Approximately 1 hr after morning milking 20 ml. milk were withdrawn aseptically from one gland of two goats and mixed with 5 μc [1,5-^{14}C]citric acid. Two ml. of this milk were then taken for determination of radioactivity and the rest injected into the cistern of the same gland via the teat canal. The gland was then gently massaged. Blood samples were taken from the 'milk' vein 1·5, 3 and 4 hr after the injection of labelled citrate. The samples were taken while the external pudic vein was being compressed manually in order to obtain pure mammary venous blood (Linzell, 1960). The animals were milked after 4 hr, oxytocin (100 mu. I.V.) being given to obtain as much of the residual milk as possible. ^{14}C in milk and plasma was determined by liquid scintillation spectrometry.

Passage of citrate from blood to milk was studied as described previously for other substances (Linzell & Peaker, 1971*c*, 1974, 1975) in two goats by the close-arterial infusion of [1,5-^{14}C]citric acid in 0·154 M-NaCl. The gland was milked 20–25 min after the start of the infusion and successive samples taken for determination of radioactivity and analysis. Milk Na, K, Cl and lactose were determined as described by Fleet, Linzell & Peaker (1972) and citrate by the method of White & Davies (1963).

In two other goats the experiment was repeated 2·5 hr after starting hourly milking (1 u. oxytocin I.V. each time).

Distribution of labelled citrate in mammary slices. Slices (0·6 mm thick) of lactating guinea-pig mammary glands were incubated at 39° C as described by Linzell & Peaker (1971*b*) for 1 hr with 0·5 μc [1,5-^{14}C]citric acid in 100 ml. bicarbonate-buffered medium of Krebs & Henseleit (1932) plus glucose (6 mM), gassed continuously with 5 % CO_2 in O_2. The volume of distribution of ^{14}C was determined as described previously.

Passage of citrate and other substances into diluted milk. In five goats approximately 4 hr after morning milking, 100 ml. sterile, isosmotic lactose (300 mM) was injected into one gland via the teat canal; the gland was then massaged. Two hr later both glands were milked and samples taken for analysis Oxytocin (100 mu. I.V.) was then given to obtain residual milk for analysis. In addition to lactose, Na, K and Cl (see above), the milk was analysed for total nitrogen (Linzell, 1967) and calcium (atomic absorption spectrophotometry).

RESULTS

Time course of secretion of milk constituents from labelled precursors

The time course of changes in specific activities of citrate, lactose and fatty acids in milk following I.V. injection of labelled glucose and chylomicrons is shown in Fig. 1. Maximum specific activities of [^{14}C]lactose were reached simultaneously at 3 hr, whereas the maximum specific activities

of [^{3}H] and [^{14}C]fatty acids in the milk triglycerides were reached at 7 hr. In the goat which was injected with [^{14}C]acetate and chylomicrons the maximum specific activities of citrate and lactose also occurred simul-

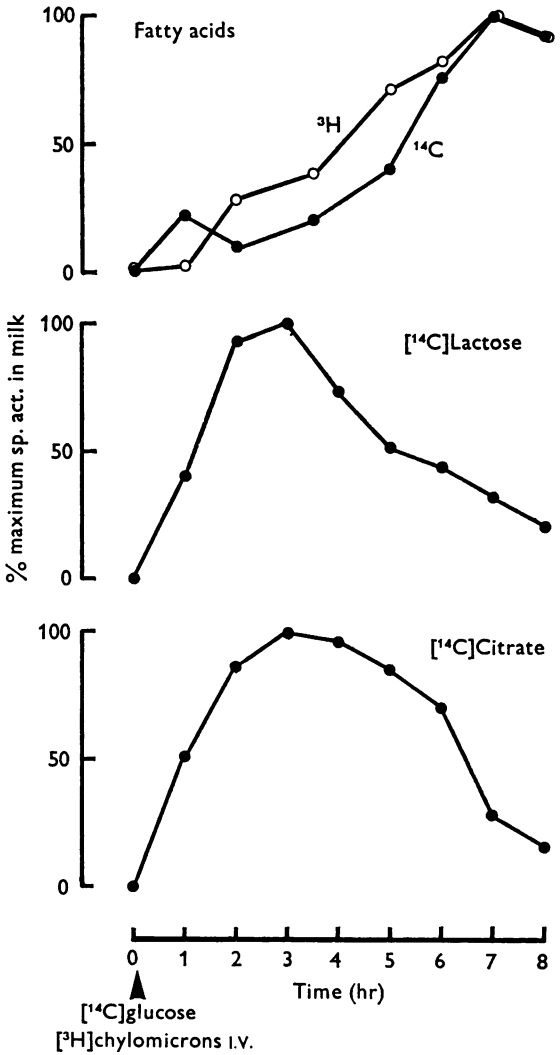

Fig. 1. Time course of the changes in specific activities (sp. act.) of fatty acids, lactose and citrate following the I.V. administration of [^{14}C]glucose and [^{3}H]chylomicrons. The goat was milked as described in the text. The graphs are standardized by expressing all points as a percentage of the maximum specific activity of that substance achieved in milk during the experiment.

taneously, in this case at 2 hr, whereas maximum labelling of the fatty acids was not reached until 3 hr later. Thus in both experiments, maxi-specific activities of citrate and lactose were reached simultaneously 2–3 hr after precursors were given and considerably earlier (by 3 and 4 hr) than the time at which maximum specific activities of the fatty acids in triglyceride were reached (Fig. 2).

By contrast, the maximum specific activities of ^{24}Na, ^{36}Cl and ^{42}K were reached within 1 hr or less of the mid point of a 14 min I.A. infusion (a short infusion was used in these experiments to prevent the rapid clearance of these isotopes, especially ^{42}K, from plasma) (Fig. 2).

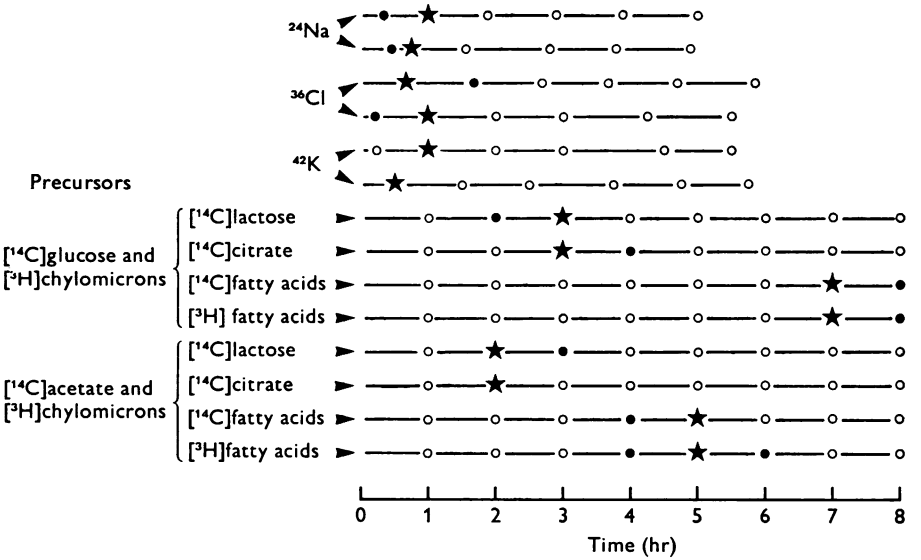

Fig. 2. Time course of changes in specific activities (sp. act.) of milk constituents following the administration of labelled precursors. Time 0 is, in the case of the labelled ions, the mid point of a 14-min I.A. infusion, and for the other substances the time of the I.V. injection of precursors. At each point the animals were milked (see text for details): ★, maximum specific activity in milk; ●, specific activity > 95% of maximum in milk; ○, < 95% of maximum in milk.

Thus, substances appearing in milk can be divided into three groups depending upon the time at which maximum specific activity is reached during frequent milking following the intravascular administration of labelled precursors: (i) those at maximum specific activity within 1 hr, i.e. Na, K and Cl in the present experiments, ^{3}HOH (Linzell & Peaker, 1971*c*), urea (Linzell & Peaker, 1971*c*), CO_2 (including carbonic acid and bicarbonate) from [^{14}C]HCO_3 (Hardwick, 1965; Linzell & Peaker, 1975) or from

[^{14}C]glucose (the late J. L. Linzell and M. Peaker, unpublished observations); (ii) those reaching maximum specific activity at 2–3 hr, i.e. citrate and lactose; (iii) those reaching maximum specific activity at 5–7 hr, i.e. milk fat.

Permeability to citrate

Milk to blood

Recovery of ^{14}C in the milk 4 hr after the introduction of [^{14}C]citrate into the cistern of the gland was 96 and 97 % in two goats. Since not all the milk can be removed by milking, these results suggest that the labelled citrate remained in the milk during this period and therefore that the mammary epithelium is virtually impermeable to citrate passing from milk to blood. Furthermore there were no significant amounts of radioactivity in mammary venous blood samples taken while [^{14}C]citrate was in the gland.

Blood to milk

In two goats [^{14}C]citrate was infused I.A. in order to maintain constant blood concentrations (Linzell & Peaker, 1971*c*, 1974, 1975). Radioactivity was determined in successive milk samples taken 20–25 min after the start of the infusion. Such milk fractions, removed at a single milking, come from different regions of the gland, the earlier fractions from the duct system and the last, obtained only after the injection of oxytocin, from the alveoli or secretory portions (Linzell & Peaker, 1971*c*).

No radioactivity could be detected in the early fractions but in the alveolar fraction ^{14}C activity was slightly above background. Calculations from these figures and the plasma ^{14}C concentration at 25 min showed that the concentration in alveolar milk was less than 0·1 % of that of the plasma. This figure is similar to that for [^{14}C]sucrose but very much lower than that for such substances as ^{3}HOH, urea, $^{14}CO_2$, ^{24}Na, ^{42}K, ^{86}Rb and ^{36}Cl (Linzell & Peaker, 1971*c*, 1974*a*, *b*). Thus these results indicate that in normal lactation the mammary epithelium is almost totally impermeable to citrate. From the pH of plasma it can be inferred that the citrate would have been almost entirely in the calciocitrate form.

By contrast, during treatment with large doses of oxytocin (1 u. every hour), the ^{14}C content of alveolar milk was 15·1 and 13·8 % of that of the plasma. During these experiments, as found previously (Linzell, 1967; Linzell & Peaker, 1971*d*), milk [Na] and [Cl] increased while [K] and [lactose] decreased; [citrate] also decreased.

We have previously suggested that exogenous oxytocin, especially in large doses, disrupts the mammary epithelium to permit the paracellular movement of relatively small molecules like lactose and ions between interstitial fluid and milk probably through 'leaky' tight junctions

(Linzell & Peaker, 1971*d*; Linzell, Peaker & Taylor, 1975). The present results indicate that citrate also can pass between blood and milk under these circumstances. Therefore the fall in the citrate concentration in milk during treatment with large doses of oxytocin, like that of lactose, can be attributed to passage down the concentration gradient from milk into blood when the paracellular route is open.

Distribution in slices in vitro

The apparent volume of distribution of [^{14}C]citrate in guinea-pig mammary slices was calculated to be $456 \pm 19{\cdot}6$ (S.E. of mean) ml./l. tissue water. This value is not significantly different from those for labelled sucrose, inulin or lactose (Linzell & Peaker, 1971*b*). Evidence was presented previously that these substances act as extracellular markers in mammary slices *in vitro*, and the results for [^{14}C]citrate indicate that this substance does not enter mammary cells to any significant extent; again it is likely that the citrate was in the calciocitrate form at the pH of the incubation medium.

Passage of milk constituents into diluted milk

In these experiments 100 ml. isosmotic lactose (300 m-osM) were injected into one gland 4 hr after morning milking. It was calculated that at the time of injection 228–380 ml. milk were present in the glands. After a further 2 hr, the volume and composition of milk were determined. The quantity of a substance entering the diluted milk was calculated in the following manner. It was assumed that no water passed osmotically into or out of the milk during the experiment (see below) and that the milk secreted during the 6 hr had the same composition as that secreted over the previous day. Therefore the expected amount of the substance was taken as the concentration $\times$ total volume after 6 hr $-$ 100 ml. (the volume of the diluent). The actual amount obtained was calculated from the concentration and total volume at the end of 6 hr; the results are shown in Fig. 3. For fat, protein, citrate and calcium, the expected and obtained quantities were not significantly different, whereas for Na, K and Cl the amounts obtained were significantly greater. In other words Na, K and Cl had entered the stored milk thus tending to restore milk concentrations of these substances to normal, whereas there was no compensatory increase for protein, citrate and calcium. Thus it can be inferred that the rate of extrusion of protein, citrate and calcium from the secretory cells continued at the previous rate even though the concentrations were lowered in milk.

As a check on the assumption that little or no water crossed the mammary epithelium osmotically during the experiment, the volume of milk obtained (total

volume after 6 hr, before oxytocin administration, minus 100 ml.) was compared with the amount expected in that time. The latter was calculated in two ways, firstly from the mean hourly yield for three days before the experiment, and secondly from the yield of the untreated gland using the R.M.Q. test for parallel variations in milk yield between the two glands (Linzell & Peaker, 1971*d*). There was no significant difference between the expected and obtained yields ($P > 0{\cdot}4$) which indicates that there was little or no water movement into or out of the diluted milk and that the rate of milk secretion was not affected by the presence of 100 ml. isomotic lactose in the gland.

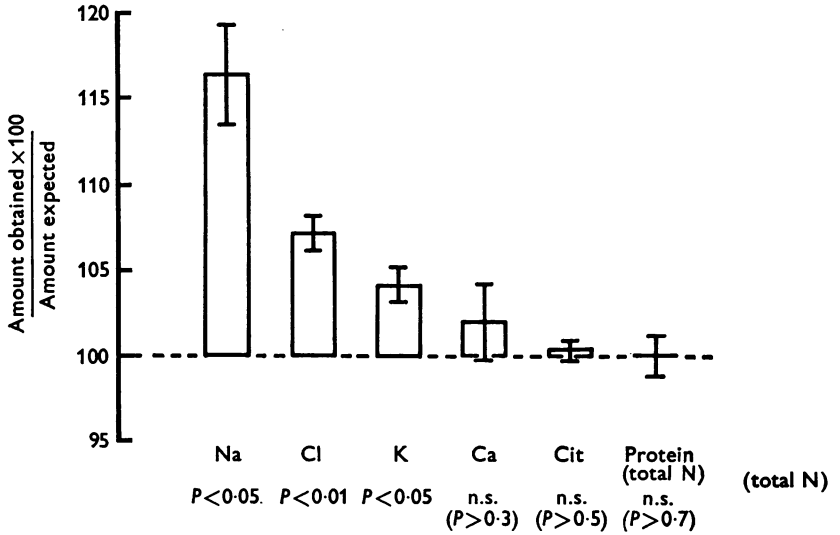

Fig. 3. Passage of milk constituents into milk diluted with 100 ml. isomotic lactose for 2 hr. The ordinate is the quantity of a substance in milk at the end of the experiment expressed as a percentage of that expected from the milk yield and previous concentration (see text for details) (mean ± S.E. of mean in five goats). Therefore substances showing a change significantly above 100 % entered diluted milk at a rate in excess of the net secretion rate. The P values were obtained by a paired *t* test between the amounts obtained and those expected, n.s. = not significant.

DISCUSSION

The results of the experiments with isototopically-labelled precursors and labelled citrate confirm the findings of Hardwick *et al.* (1963*b*) that milk citrate is synthesized in the mammary gland, citrate carbon being derived from both glucose and acetate.

It is generally believed that lactose and specific milk proteins are secreted into milk by the Golgi apparatus, the former being synthesized within the Golgi apparatus, the latter on the endoplasmic reticulum. Secretory vesicles then move towards the apex of the cell and fuse with

the apical membrane to release their contents by exocytosis (see Linzell & Peaker, 1971*a*). This hypothesis fits our findings that the apical cell membrane is impermeable to lactose because formation of lactose in the Golgi apparatus is equivalent to its formation outside the cell (Linzell & Peaker, 1971*a*, *b*, *d*).

Other evidence that the modes of secretion of protein and lactose are similar can be deduced from the work of Wood, Joffe, Gillespie, Hansen & Hardenbrook (1958) who followed the time course of specific activities in milk of lactose, serine (from casein) and fatty acids following the I.A. injection of [^{14}C]glycerol in a cow. Peak specific activities of both lactose and serine were reached at approximately 2 hr, whereas the maximum for fatty acids was not reached until 4–5 hr. Therefore it can be argued that protein and lactose are secreted by a similar route whereas milk fat, which accumulates as droplets in the cell, is, as ultrastructural evidence shows, secreted by a different mechanism (see Linzell & Peaker, 1971*a*). It appears that the time at which labelled products are secreted after the administration of labelled precursors may indicate the route of secretion by the alveolar cell. Thus substances like ^{3}HOH, Na, K, Cl, urea and CO_2 (from plasma CO_2 or from oxidized substrates) which are thought to enter milk by passage through cell membranes (Linzell & Peaker, 1971*a*, *b*, *c*; 1975) are at maximum specific activity in 1 hr or less, whereas lactose and citrate which do not permeate the membranes and are secreted by Golgi vesicles reach maximum specific activity in 2–3 hr, and milk fat secreted as globules reaches maximum specific activity in 5–7 hr.

A similar argument can be advanced to account for the time course of citrate secretion. It is suggested that citrate, as well as lactose and specific milk proteins, is formed in, or accumulated by, the Golgi apparatus and that vesicles carry citrate to the lumen of the alveolus. The permeability studies *in vivo* and *in vitro*, like those with lactose (Linzell & Peaker, 1971*a*, *b*, *d*), suggest that the cell membranes are virtually impermeable to citrate in the forms normally present in extracellular fluid (probably calciocitrate$^-$) or milk (probably calciocitrate$^-$ and $HCit^{2-}$) and that citrate formed in the cell must be packaged in an effectively extracellular site for extrusion by exocytosis.

The results of the experiments in which milk in the gland was diluted with isosmotic lactose indicate that citrate, like protein, is secreted at a steady rate and does not respond to an altered electrochemical gradient across the apical cell membrane like the permeating simple ions Na, K and Cl. Therefore this evidence from 'bulk flow' studies also indicates that the passage of citrate into milk is not a case of simple diffusion according to the electrochemical gradient across the apical cell membranes, which is in agreement with the permeability studies with isotopically-labelled

citrate. It thus appears that citrate, once secreted into milk, is held at high concentrations by virtue of the impermeability of the alveolar and duct epithelia to this substance, but that if a paracellular route is open as a result of treatment with high doses of oxytocin or in other physiological or pathological conditions (see Peaker, 1975) then citrate is lost from milk by passage back into the blood.

The results on the impermeability of mammary cell membranes to citrate are in agreement with studies on the red cell membrane (see Höber, 1936, for example) and it would appear that the relatively large anions, Cit^{2-} and calciocitrate$^-$, cannot permeate routes available to the smaller anions.

If citrate is secreted by the Golgi route we must consider how citrate gets into the Golgi apparatus. Comparative studies indicate that in species where the milk citrate concentration is low (rat) citric cleavage enzyme (ATP-citrate lyase) is present in the cytosol of mammary homogenates, whereas in ruminants which have high milk citrate concentrations, the levels of this enzyme are very low (see Bauman, Mellenberger & Derrig, 1973). This correlation would argue against the synthesis of citrate within the Golgi apparatus because it would appear that milk citrate is derived from that in the cytosol. Similarly, Hardwick (1966), in isotopic studies on goat mammary glands, concluded that no large pool of citrate in mammary tissue had a markedly different specific activity from that of milk citrate. Calculations from the milk citrate content of rat and ruminant milk and the calculated concentrations of citrate in the cytosol of these species (Gumaa, Greenbaum & McLean, 1971; Gumaa, McLean & Greenbaum, 1973) indicate that the concentration in milk is approximately sixteen times that of intracellular fluid. Therefore the lower milk [citrate] in the rat is correlated with a lower cytosol [citrate]. Thus this evidence suggests that citrate is concentrated rather than synthesized by the Golgi apparatus.

For citrate to be transported into the Golgi apparatus there appear to be three possible mechanisms:

(i) Passage as unionized citric acid and an alteration in pH to trap citrate in an ionized form. The pH necessary would however seem to be too low, the pK_a of the dissociation being 3·13 at 25° C.

(ii) Diffusion of citrate into the Golgi apparatus to a concentration determined by the electrochemical gradient across the Golgi membrane. This would demand the permeability of the Golgi membrane to citrate in an ionized form, and the potential gradient required to achieve a sixteenfold concentration difference of $HCit^{2-}$ or Cit^{3-} probably exists (the late J. L. Linzell and M. Peaker, in preparation).

(iii) A carrier-mediated transport of citrate into the Golgi apparatus. This mechanism would not be without precedent since a carrier mechanism is thought to be involved in citrate movements across the mitochondrial membrane (see Munn, 1974).

Until these questions are resolved it is not possible to attempt an explanation of the rapid rise in the citrate concentration of the mammary secretion at about the time of parturition in goats, cows and women (Peaker & Linzell, 1975; Fleet *et al.* 1975).

Since several groups of workers have now succeeded in obtaining by centrifugation a relatively pure Golgi fraction from mammary homogenates (see, for example, Keenan, Morré & Cheetham, 1970; Keenan, Huang & Morré, 1972) it would clearly be desirable to analyse such fractions for their citrate content and to study citrate movements across the Golgi membrane.

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