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Use of an ion-selective electrode to determine free Ca ion concentration in the milk of various mammals

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Milk is a heterogeneous fluid in which the colloidal phase is homogeneously dispersed in the aqueous phase. Calcium is partitioned between the colloidal and aqueous phases and is in complex electrochemical equilibrium with several major milk components. In human and bovine milk, calcium is mainly distributed between the aqueous and casein micelle in the colloidal phases (Holt & Jenness, 1984; Neville et al. 1994). Caseins form a complex micelle structure that contains approximately 25 000 phosphorylated monomers that react with calcium phosphate complexes in the milk to bind 20–40 mole calcium per mole casein (Holt & Jenness, 1984; Neville et al. 1994). Thus, the distribution of calcium between the colloidal and aqueous phases appears to be governed by the level of casein in the milk (Holt & Jenness, 1984; Neville et al. 1994). In human milk, the casein level is low; ~25% of calcium is associated with casein, whereas in cows and goats the corresponding figure is higher at ~65%. In rats, casein levels are among the highest in mammals and 95% of calcium is associated with casein (Neville et al. 1994). In the aqueous phase, calcium is divided among ionic calcium (Ca^{2+}), calcium citrate and calcium phosphate. Calcium citrate and calcium phosphate constitute most of the aqueous calcium in bovine milk in contrast to less than 50% in human milk (Holt & Jenness, 1984; Neville et al. 1994). As the concentration of Ca^{2+} in milk appears to be essential in preserving the integrity of the mammary tight junctions during lactation (Neville & Peaker, 1981), it is important to follow its concentration precisely in mammary secretions in different stages of the reproduction cycle.

The concentration of Ca^{2+} in milk can be determined with a calcium-selective electrode (Allen & Neville, 1983). The presence of a metal-chelator complex in the solution affects the ionic activity of the soluble ion and, consequently, the response of an ion-selective electrode is not proportional to ion concentration (Kim & Padilla, 1978; Schoenmakers et al. 1992). Determination of ion concentration in solutions that contain chelators requires, therefore, special correction procedures (e.g. Kim & Padilla, 1978) or elimination of the chelator from the solution. As noted above, caseins are powerful biological chelators. Nevertheless, to the best of our knowledge, potential effects of caseins on determination of Ca^{2+} concentration in milk have not been considered. The aim of this study was to verify that caseins in the milk of various mammals interfere with the determination of Ca^{2+} concentration with an ion-selective electrode, and to evaluate several ways of correcting for the interference.

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Materials and Methods

Fresh milk samples from six cows, six goats, and six sheep were taken from animals belonging to experimental herds of the Agricultural Research Organization. Sufficient milk for analysis was accumulated from several lactating laboratory mice. Human milk was taken from a healthy volunteer. All milk samples were defatted (Shamay et al. 2000), and the skim milk was analysed, within 2–3 h of sampling, for Ca^{2+} concentration as described below. Casein concentration (the difference between total protein and whey contents of skim milk) and total calcium concentrations (by Inductively Coupled Plasma – Atomic Emission Spectrometry) were determined in the defatted milk (Shamay et al. 2000).

Ca^{2+} was measured with a Ca^{2+}-selective electrode (MeterLab pH/4201, a Portable pH Meter equipped with selective electrode 813D-12 and reference electrode: REF251; Radiometer Analytical, Denmark) at 25±1 °C. Ca activity is affected by ionic strength in the test solution. According to the extended Debye-Huckle equation, the ionic strength in milk represents weak interference. In order to measure standards and samples under the same conditions, we added KCl to both standards and samples to obtain high ionic strength level compared with the sample ionic strength. The electrode response was calibrated by running $CaCl_2$ solutions containing 0·1 M-KCl with Ca^{2+} concentrations ranging from 10^{-1} M to 10^{-4} M. Standards were measured a second time at the end of sample measurements to ensure that no drift in the

electrode response occurred during the measurements. To verify that the determination of Ca^{2+} in milk was not affected by non-specific interaction (adsorption) between protein and the electrodes, the standards were measured in the presence of 2%, 4%, 6%, and 8% of bovine serum albumin (a non-chelating protein).

Concentration of Ca^{2+} in milk was determined according to the following four procedures:

1) *Uncorrected reading* The electrode was immersed in defatted milk, and the output signal of the electrode in mV was converted to Ca^{2+} concentration from the calibration curve.

2) *Multiple addition method* This is the method recommended by the manufacturer to correct for a chelating effect. Aliquots of 100 µl 0·1 M-$CaCl_2$ were added to 10 ml milk until the electrode response was constant; usually four additions were required to get a constant response. Ca^{2+} concentration was determined according to the following equation, which derives from the Nernst equation:

$$Csmp = \frac{Cstd \times Vstd}{(Vstd + Vsmp) \times 10^{(E_1 - E_0)/S} - Vsmp}$$

where:

Csmp = Ca^{2+} concentration (mmol) in the milk sample

Cstd = Ca^{2+} concentration (mmol) in the standard

Vstd = Standard volume (ml)

Vsmp = sample volume (ml)

E_0 = Electrode reading of sample before the addition of standard (mV)

E_1 = Electrode reading of sample after the addition of standard (mV)

S = Electrode sensitivity; the change in electrode potential (mV) in response to a 10-fold change in Ca^{2+} activity.

3) *Equilibration dialysis* Defatted milk samples (1 ml) in a dialysis bag (molecular cut-off 3500) were equilibrated with 30 ml water at 4 °C for 24 h while being stirred. Ca^{2+} concentration was determined in the equilibrating fluid as described in method 1. The analysis was carried out in triplicate.

4) *Ultrafiltration* Defatted milk samples (5 ml) were centrifuged at 1000 ***g*** for 1 h in a ultrafiltration unit (Millipore-Amicon, USA with molecular cut-off 10 000). The filtrate was diluted 1:10, and Ca^{2+} concentration was determined as described in method 1. The analysis was carried out in triplicate.

Statistical differences between the results obtained by the four different methods within species were evaluated by paired *t* test between each two methods followed by ranking them.

Results and Discussion

As expected, a plot of the electrode response (mV) against Ca^{2+} concentration on a log-log basis gave a straight line with an r^2 value of 0·98–1. A 10-fold increase in Ca^{2+} activity should result in an increase of 29·6 mV in the electrode response according to the Nernst equation, which was close to the experimental value of 28·2. The electrode readings obtained when standards contained 2%, 4%, 6%, and 8% albumin were similar (±5%) to those obtained when standards were run in protein-free solutions. The lack of response to non-chelating protein (albumin) suggests that the interfering effect of casein relates to its chelating properties.

In the multiple addition method, pH dropped by 0·1 unit after four additions of $CaCl_2$ (from 6·6 to 6·5). This change is too small to affect electrode sensitivity, which is reported by the manufacturer to be stable from pH 3–12. Acidification may cause dissociation of calcium from the

Table 1. Procedures for determination of Ca^{2+} concentration in the milk of cows, goats and sheep, using an ion-selective electrode

	Method of Determination†							
Species	UC	SE	RA	SE	ED	SE	UF	SE
Cow	1·81[a]	0·14	3·17[b]	0·13	2·98[b]	0·28	2·93[b]	0·33
Goat	1·67[a]	0·37	3·48[b]	0·27	3·50[b]	0·34	3·69[b]	0·48
Sheep	2·01[a]	0·28	3·98[b]	0·11	4·22[b]	0·46	4·17[b]	0·41

† UC = Uncorrected method; RA = Repeated Addition method; ED = Equilibration Dialysis method; UF = Ultrafiltration method

Means within a row without common superscripts differ significantly ($P < 0·01$)

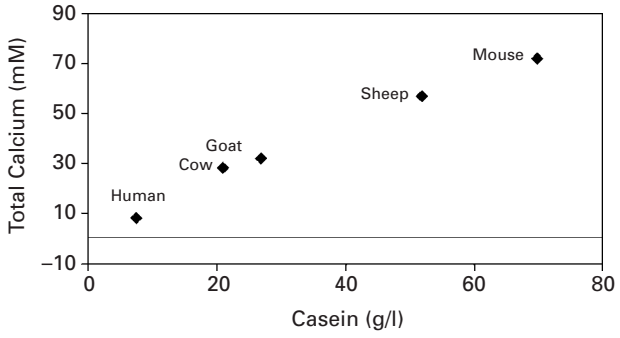

Fig. 1. Interrelationship between the concentration of total calcium and the concentration of casein in the milk of cows, goats, sheep, humans and mice.

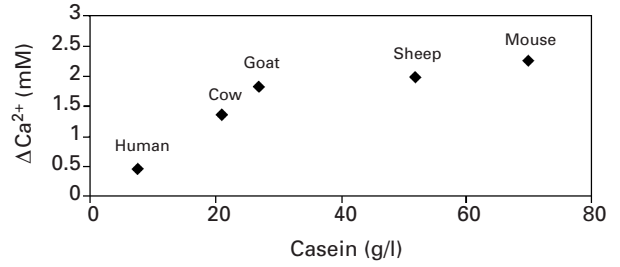

Fig. 2. Interrelationship between the difference between uncorrected and corrected (multiple addition procedure) Ca^{2+} concentration (Δ Ca^{2+}) and the concentration of casein in the milk of cows, goats, sheep, humans and mice.

casein micelle (Holt & Jenness, 1984). To test for this possibility, we measured Ca^{2+} concentration in milk samples at pH 6·6 (adjusted by adding NaOH). The results were essentially similar to those obtained with the regular $CaCl_2$-0·1 M-KCl standards (pH 6·5). This suggests that a drop in pH of 0·1 unit is too small to cause release of measurable amounts of calcium from the casein micelle.

Total calcium concentrations in milk in the tested species ranged from 8 mM in human milk to 71 mM in the mouse and were positively related to casein concentration (Fig. 1), supporting previous results (Neville et al. 1994). Despite the large interspecies fluctuation in total calcium concentration, Ca^{2+} concentrations in milk in these species ranged from 3·2–4·2 mM (Table 1). This range is similar to the range of Ca^{2+} concentration in extracellular fluid of mammals, which suggests that maintaining Ca^{2+} concentrations within these limits may be important for mammary gland function.

The present results clearly show that casein markedly interferes with ionized calcium (Ca^{2+}) determination in mammals (Table 1). The interfering effect of casein was positively related to casein concentration (Fig. 2), so that the uncorrected method underestimated the values obtained by one of the corrected methods (multiple addition method) by 43% in cows and by ~50% in goats and sheep (Table 1). Our value for uncorrected Ca^{2+} concentration of 2·7 mM in human milk are consistent with reported values of 2·8 (Allen & Neville, 1983) and 2·3–2·9 (Kent et al. 1992). Our value for uncorrected Ca^{2+} concentration of 2 M in cows is also consistent with reported values (Neville & Peaker, 1981; Neville et al. 1994). Thus, our results suggest that most of the values for milk Ca^{2+} concentration reported hitherto in the literature are grossly underestimated.

The three correction procedures yielded similar results for all species tested, suggesting that the three methods are suitable for ameliorating the suppressing effect of casein. The multiple addition technique is recommended as the method of choice in respect of ease of application, cost and time. In the case of cow and sheep samples, the SE was considerably lower than that found with the other two methods.

The equilibration dialysis method is easy to apply, relatively cheap, but relatively time-consuming. We found that an equilibration period of 12 h resulted in values lower than those obtained with the other two methods, most likely because of a lack of equilibration. On the other hand, an equilibration period of 36 h resulted in values higher than those obtained with the other two methods, most likely because of dissociation of Ca^{2+} from the casein micelle. Since the time needed for optimal equilibration was determined empirically, it is suggested that the appropriateness of the duration of the dialysis process be re-examined in the following cases: (i) if milk samples are not fresh, (ii) if the samples come from other forms of mammary secretion, such as colostrum, and mammary secretion during involution, and (iii) if the sample comes from species not tested in this study.

The ultrafiltration method appears to be the most versatile as it is appropriate for most types of mammary secretion. This method is easy to apply, relatively rapid, albeit relatively expensive when large number of samples have to be analysed.

References

Allen JC & Neville MC 1983 Ionized calcium in human milk determined with a calcium-selective electrode. *Clinical Chemistry* **29** 858–861

Holt C & Jenness R 1984 Interrelationships of constituents and partition of salts in milk samples from eight species. *Comparative Biochemistry and Physiology* **77A** 275–282

Kent JC, Arthur PG, Retallack RW & Hartmann PE 1992 Calcium, phosphate and citrate in human milk at initiation of lactation. *Journal of Dairy Research* **59** 161–167

Kim YS & Padilla GM 1978 Determination of free Ca ion concentrations with an ion-selective electrode in the presence of chelating agents in comparison with calculated values. *Analytical Biochemistry* **89** 521–528

Neville MC & Peaker M 1981 Ionized calcium in milk and integrity of the mammary epithelium in the goat. *Journal of Physiology, London* **313** 561–570

Neville MC, Keller RP, Casey C & Allen JC 1994 Calcium partitioning in human and bovine-milk. *Journal of Dairy Science* **77** 1964–1975

Shamay A, Shapiro F, Barash H, Bruckental I & Silanikove N 2000 Effect of dexamethasone on milk yield and composition in dairy cows. *Annales de Zootechnie* **49** 343–352

Schoenmakers TJ, Visser GJ, Flik G & Theuvenet AP 1992 CHELATOR: an improved method for computing metal ion concentrations in physiological solutions. *Biotechnique* **12** 870–874; 876–879