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Ultrafiltration as a valuable method in calcium-binding studies with milk proteins

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From the ultrafiltration patterns of calcium chloride solutions without protein and from the calcium-binding patterns of whole casein and whole casein + β-lactoglobulin (β-lg), it was found that, during ultrafiltration (UF), calcium was rejected by the membrane. The rejection of calcium was independent of the applied pressure but it was affected by the presence of protein(s). The higher the calcium concentration in the feeding solution the lower was the rejection coefficient. The presence of lactose slightly affected the rejection of calcium. The retention coefficients (R) of UF membranes used for β-lg and α-lactalbumin (α-la) were determined. Both types of membranes showed high retention coefficients ($R > 0.99$) for these proteins. The amount of calcium bound to casein increased with increasing pressure. By extrapolation to zero pressure the true amount of calcium bound to protein can be calculated. Before any binding study, the ultrafiltration profile of the system and the membrane to be used should be studied.

INTRODUCTION

Many separation techniques have been employed in binding studies with proteins, including equilibrium dialysis, gel filtration, ultracentrifugation, the use of selective ion electrodes and ultrafiltration (UF).

In equilibrium dialysis, 24–72 h are usually required to establish equilibrium conditions. This method is usually carried out at 3°C to avoid growth of microorganisms. It requires both space and facilities. Furthermore, the results obtained by dialysis at low temperatures are not applicable to milk at normal ambient temperatures (Davies & White, 1960). In ultracentrifugation methods the supernatant contains all the soluble (serum) proteins and a trace of the calcium caseinate complex, so that ultracentrifugation is not a reliable method for milk protein-binding studies. Calcium selective electrodes do not respond to the very small changes in calcium concentrations likely to be encountered in calcium-binding studies to many proteins and gel filtration frequently results in contamination of the protein solution by the salts of the elution buffers (Hancher & Ryon, 1973). On the other hand, ultrafiltration is relatively convenient, simple and rapid compared with other methods employed in binding studies. Since the recent introduction of new ultrafiltration membranes, which are polymeric, non-cellulosic, mainly non-ionic and biologically inert with a high degree of selectivity, ultrafiltration has become a valuable aid in the separation of micromolecules from macromolecules, with exceptional throughputs at modest pressures. Although the UF technique has been used by many authors (Toribara *et al.*, 1957; Farese *et al.*, 1970; Ryan & Hannam, 1971; Crawford *et al.*, 1972; Hancher & Ryon, 1973; Spector *et al.*, 1973; Yap & Schaffer, 1977; Baumy & Brule, 1988), it has neither been used in binding studies with milk proteins nor evaluated for its applicability to such studies.

The present paper deals with UF as a technique for studying the binding of milk proteins to calcium ions; the continuous ultrafiltration method and its binding

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profiles, the effect of ultrafiltration pressure on the extent of calcium binding to milk proteins and the permeability of membranes to proteins and calcium were studied.

MATERIALS AND METHODS

Apparatus

In the present studies the continuous ultrafiltration (diafiltration or equilibrium ultrafiltration) method has been used. The method is illustrated diagrammatically in Fig. 1. The protein sample, with appropriate concentration of calcium, is put into the ultrafiltration (UF) cell. The reservoir is filled with calcium solution of the same concentration as the sample solution. Reservoir and cell compartment are then equally pressurized by nitrogen gas through the valves 1 and 2. The gas valve 1 of the cell is closed and, as the system operates, at constant stirring, the liquid (filtrate), which is continuously discharged through the UF membrane, is replaced by an equal volume from the reservoir through the valve 3, so restoring the pressure in the cell. Thus, the volume of the sample in the UF cell remains essentially constant during the run. The system should be pressurized to deliver ultrafiltrate at a rate of 1% or less of the sample volume per minute. This permits binding equilibrium between calcium and protein to be established. Successive 5 ml filtrates are collected and analysed for calcium. When the concentrations of calcium in filtrate and feeding solutions are essentially the same, the equilibrium of binding is established and the run is completed. The type of UF equipment used was the multicell micro-ultrafiltration system, model MMC, with a common 800-ml capacity reservoir for simultaneous diafiltration of up to eight different samples. Each sample can be operated individually, except for the common stirring-speed control. The maximum capacity of each cell is 10 ml.

In one case, when the protein retention coefficient of UF membranes was determined, the discontinuous (batchwise) UF technique was used; in this case the 10 ml stirred single cell, model No, 12, was used, which differs from the previous ones in that the reservoir is omitted and it has only one UF cell instead of eight. The cell is charged with the sample and then pressuized with nitrogen gas. When 5 ml of filtrate has been collected, the unit is depressurized and a further 5 ml of calcium chloride solution is added to the cell. This procedure is repeated until the calcium concentrations in the filtrate and feeding solution are essentially the same, i.e. binding equilibrium is established.

The types of UF membranes tested were the Diaflo® UM 10 and PM 10, each 25 mm in diameter with a nominal molecular weight cut off of 10000. Both types of equipment and membrane used in these experiments were purchased from Amicon (Lexington, Massachusetts, USA). Whole casein, β-lg and α-la were selected to evaluate the method because all of them were used in a series of calcium-binding experiments (Pappas & Rothwell, 1991). Before UF, they were treated as was required in these experiments. They were prepared as described by Pappas (1979). Different concentrations buffered in 3 mmol litre^{-1} sodium barbital, pH 7·00, were used. Calcium in ultrafiltrates and protein solution was determined according to Vogel (1962) and Ntailianas and Whitney (1963), respectively. The protein content in ultrafiltrates was determined by absorption at λ = 280 nm (Layne, 1957). All the chemicals were of analytical grade. The UF pressures applied were 68·9, 103·4, 137·9, 241·1 and 275·8 kPa (10, 15, 20, 35 and 40 psi, respectively).

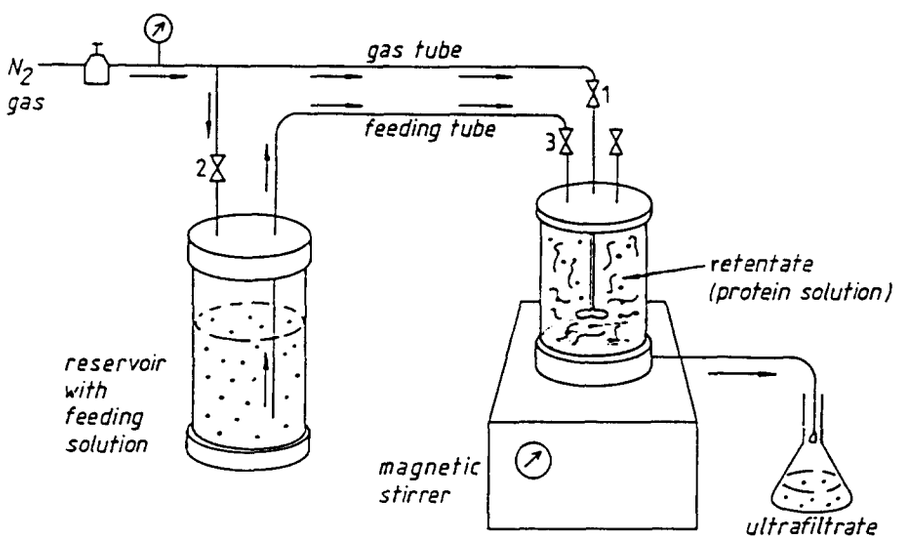

Fig. 1. Outline of a continuous ultrafiltration system.

Principles underlying continuous ultrafiltration

The continuous ultrafiltration (diafiltration) technique was first described by Blatt *et al.* (1968). They provided a general mathematical model to describe the process involved in feeding a ligand solution, i.e. $CaCl_2$, into a UF cell and to relate the effluent ligand concentration to effluent volume.

When a fixed volume of water is placed in the filtration cell and the reservoir is charged with the ligand solution of known concentration, then the concentration of the ligand in the filtrate is related to the filtrate volume by the following two equations (eqns (1) and (2)), both given by Blatt *et al.* (1968),

$$\ln \frac{C_f}{C_f - C} = \frac{V - V'}{\bar{V}_0} \tag{1}$$

where C_f is the ligand concentration in the reservoir (feeding solution), in mol litre^{-1}; C is the ligand concentration in the filtrate, in mol litre^{-1}; V is the cumulative filtrate volume, in litres; V' is the apparent void volume of the system, in litres (comprising the membrane, sintered plastic membrane support and connecting tubing); and $\bar{V}_0$ is the average sample volume during the run, in litres (quantitatively equal to the initial sample volume charged into the cell, since no changes in volume occur during the run).

Equation (1) can be written:

$$\log_{10} \frac{C_f}{C_f - C} = \frac{1}{2{\cdot}303\,\bar{V}_0} V - \frac{V'}{2{\cdot}303\,\bar{V}_0} \tag{2}$$

Plotting $\log_{10} [C_f/(C_f - C)]$ against V a straight line should be obtained. The slope of this line is $1/2{\cdot}303\bar{V}_0$, from which the values of $\bar{V}_0$, the average sample volume, may be calculated. The value of $-V/2{\cdot}303\bar{V}_0$ is the intercept of the line of the $\log_{10} [C_f/(C_f - C)]$ axis. From the intercept the value of V', the apparent void volume, can also be calculated.

Equations (1) and (2) are also applicable when, instead of water, a protein solution is placed in the filtration cell, provided that the protein is completely retained by the membrane and the low molecular weight solute (microsolute) is not. However, when the microsolute is rejected by the membrane, the rejection (reflection) coefficient σ, can be estimated by

$$\sigma = 1 - \frac{C}{C'} \tag{3}$$

where C is the microsolute concentration in the ultrafiltrate and C' is the microsolute concentration in the sample cell (*retentate*). It is obvious that, when the membrane is completely permeable to microsolute, σ should be 0·0. For the case in which σ is not equal to zero, eqn (2) becomes

$$\log_{10} \frac{C_f}{C_f - C} = \frac{1 - \sigma}{2{\cdot}303\bar{V}_0} V - \frac{(1 - \sigma)V'}{2{\cdot}303\bar{V}_0} \tag{4}$$

In this case the slope of the line will be given by $(1 - \sigma)/(2{\cdot}303\bar{V}_0)$. It may be concluded that if the average sample volume, $\bar{V}_0$, calculated from the slope of eqn (2), is significantly larger than the actual experimental value, solute rejection may be the cause. Similarly, if the calculated average sample volume in an experiment involving protein is different from the calculated average sample volume in the control without protein, then, again, the cause could be the increased rejection of microsolute, due to the presence of protein.

Calculations

In the continuous ultrafiltration method, we now consider the calculations that are applied to determine the amount of calcium bound to protein. There are two experimental cases: in the first case, the protein sample initially added to the UF cell contains no calcium; in the second case, the protein sample contains calcium, usually of the same concentration as that of feeding solution.

If it is desired to measure the amount of calcium bound up by the protein at any stage of the ultrafiltration process—for example, after n successive fractions of filtrate have been collected—and if the protein sample added into UF cell does not contain calcium, the following simple mass equation around the cell may be applied:

Amount of calcium bound =
Total amount of calcium passed from the reservoir
– amount of unbound (free) calcium in the protein sample
– amount of calcium passed in the filtrate

or

$$C_b = V_f C_0 - V_0 C_{fn} - V_f - V_f C_{ave} \tag{5}$$

where C_b is moles of calcium bound to the amount of protein(s), in grams, present in 1 litre of the solution at the time of sampling; V_f is the cumulative volume of successive ultrafiltrates, collected up to the time of sampling, in litres; C_0 is the concentration of calcium in the reservoir solution, in mol litre^{-1}; C_{ave} is the mean calcium concentration of the filtrates, in mol litre^{-1}; V_0 is the volume of sample, in litres (0·010 litre in this study); and C_{fn} is the concentration of calcium in the nth fraction of filtrate, in moles litre^{-1}.

In the case where the protein sample contains calcium at the same concentration as the feeding solution, a case which also applies in this study, the factor V_0C_0 (moles of calcium initially present in the protein sample volume) must be added to the right-hand side of eqn (5), which then becomes

$$C_b = V_f C_0 + V_0 C_0 - V_0 C_{fn} - V_f C_{ave} \tag{6}$$

Further, if it is desired to find the total calcium-binding capacity of the protein(s), C_{bm}, i.e. the maximum

amount of calcium bound to protein(s), under the experimental conditions, the UF experiment must be allowed to reach binding equilibrium; that is, the point where the calcium concentration of the ultrafiltrate is, essentially, equal to that of the feeding solution. Then, C_{bm} is simply given by the difference between the concentration of total calcium (bound and free), C_t, and the concentration of free (inbound) calcium, C_f, in the cell content, in mol litre^{-1}:

$$C_{bm} = C_t - C_f \tag{7}$$

where C_{bm} is moles of calcium bound to the amount of protein(s), in grams, present in a litre of sample solution at the binding equilibrium state.

Because equilibrium conditions in calcium binding are established, the free calcium concentration in the cell content is essentially equal to the concentration of calcium in the filtrate. Therefore, C_f in eqn (7) can be replaced by the concentration of calcium in the filtrate. This concentration, as well as C_t, is easily determined.

Equation (7) applies when calcium is not rejected by the membrane. When calcium is rejected, eqn (7) becomes

$$C_{bm} = C_t - \frac{C_f}{1 - \sigma} \tag{8}$$

where σ is the rejection coefficient for calcium by the membrane, as defined by eqn(3).

Should a change occur in the sample volume during ultrafiltration, eqn (8) is modified to

$$C_{bm} = \left(C_t - \frac{C_f}{1 - \sigma}\right)\frac{V_f}{V_0} \tag{9}$$

where V_0 is the initial sample volume, in litres; V_f is the final sample volume, in litres.

If the molar binding ratio (= the moles of calcium bound to one mole of protein), r, is required, the following relationship is applied, assuming no calcium rejection,

$$r = \frac{C_t - C_f}{C_p} \tag{10}$$

where C_p is the concentration of protein originally charged to the sample cell, in moles litre^{-1}, and C_t and C_f are given in eqn (7).

If the molecular weight of the protein is not known, the calcium bound is usually expressed in moles per 10^5 grams of protein, r'. In such a case, eqn (10) becomes

$$r' = (C_t - C_f)\frac{10^5}{C_g} \tag{11}$$

where C_g is the protein(s), in grams, present in 1 litre of sample solution.

Equation (10) and (11) may be corrected for any rejection of calcium and any change in the sample volume during UF, as previously.

RESULTS AND DISCUSSION

Evaluation of the ultrafiltration system

Ultrafiltration patterns of calcium chloride solution without protein (control experiments)

In these experiments, membranes UM10 DIAFLO were used. The cells of the ultrafiltration equipment, Model MMC, were charged with 10·0 ml of 10 mmol litre^{-1} calcium chloride solution, buffered in 3 mmol litre^{-1} sodium barbital, pH 7·00. The same solution was placed in the reservoir. Pressures applied were 68·9, 103·4, 137·9 and 275·8 kPa (10, 15, 20 and 40 psi, respectively). The calcium content of the ultrafiltrate was determined at 5 ml intervals.

Plotting the values $\log_{10}[C_f/(C_f - C)]$ calculated from the experimental data found for the different pressures, against V (eqn (2)) assuming zero calcium rejection, Fig. 2 is obtained. From the slopes of these lines the apparent sample volumes, V_0, in millilitres, were calculated and found to be 17·0, 18·0, 19·8 and 18·3 ml for pressures 68·9, 103·4, 137·9 and 275·8 kPa, respectively. That the apparent average volumes of samples calculated from the ultrafiltration data are significantly larger than the actual experimental value of 10 ml

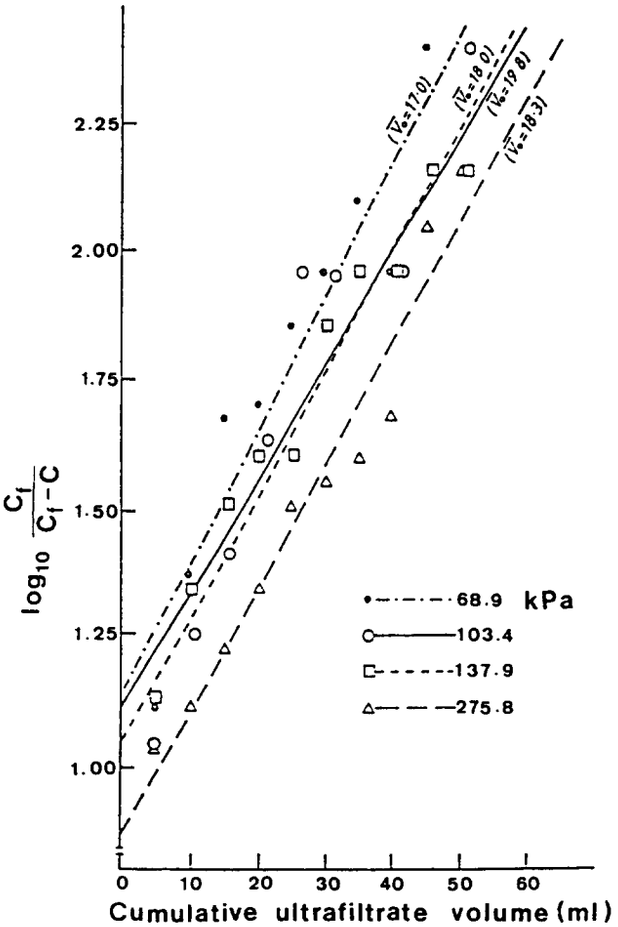

Fig. 2. Continuous ultrafiltration patterns of 10 mmol litre^{-1} $CaCl_2$ at different pressures (least-square's lines).

indicates that there is some degree of rejection of calcium by the membrane inside the cell. Another conclusion which may be drawn from the data is that, since the slopes of the UF lines calculated at different pressures (Fig. 2) are similar, the rejection of calcium in experiments without protein is independent of the applied pressure.

Similar results were also obtained by Blatt *et al.* (1968) applying the UF technique with NaCl, methyl orange and $CaCl_2$ at 50 psi (344·7 kPa).

Ultrafiltration calcium-binding pattern of whole casein + β-lactoglobulin

The UF cell Model MMC and UF membranes of UM 10 DIAFLO type were used.

Ten millilitres of a 2·5% solution of whole casein with 0·3% β-lg, previously heated at 95°C for 30 min in 20 mmol litre^{-1} $CaCl_2$, buffered in 3 mmol litre^{-1} sodium barbital, pH 7·00, was used. The same calcium chloride solution, buffered also in 3 mmol litre^{-1} sodium barbital, pH 7·00, was placed in the reservoir. A pressure of 137·9 kPa (20 psi) was applied. A control containing no protein was run simultaneously. Successive 5 ml filtrates were collected and analysed for calcium. Applying eqn (3), the results are shown in Fig. 3. As the volume of the sample in the cell did not actually change during ultrafiltration, the differences for the sample volume, between the actual (10 ml) and the calculated (9·0 and 9·8 ml for control and whole casein + β-lg samples, respectively) values, indicate that a rejection of calcium by the membrane took place in both experiments. However, since these two calculated values (9·0 and 9·8) differ from each other it would appear that the rejection of calcium is affected by the presence of protein. Further experiments were carried out to determine the magnitude of this effect.

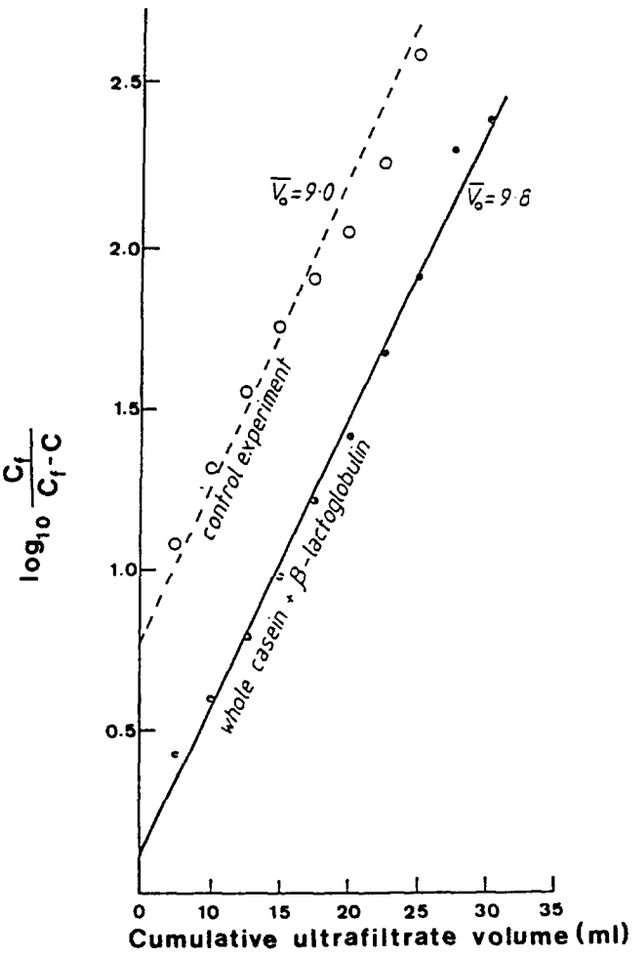

Fig. 3. Calcium rejection and continuous ultrafiltration binding equilibrium of 2·5% whole casein + 0·3% β-lactoglobulin in 20 mmol litre^{-1} $CaCl_2$ at pH 7·00 and 137·9 kPa (20 psi) (least-square's lines).

If the calcium concentrations of the ultrafiltrates are plotted against the cumulative ultrafiltration volume, the curves shown in Fig. 4 are obtained. The lower curve represents the binding kinetics of calcium to whole casein + β-lg, while the upper curve is the rejection curve of calcium in the absence of proteins (control). It can be seen from the control curve that calcium is rejected by the membrane inside the cell. The rejection was high at the beginning of the run. As UF proceeded, the rejection of calcium reached a state of equilibrium and remained, essentially, constant thereafter. The concentration of calcium in the ultrafiltrates approached (asymptotically) that of the feeding solution (horizontal line in Fig. 4). This equilibrium was achieved when three times the sample volume of ultrafilter (i.e. 30 ml) had passed through the membrane. This means that, beyond this point, the rejection of calcium remains constant whether or not proteins are present in the UF cell. It can also be seen from the binding-kinetics curve, that calcium binds to whole casein + β-lg continuously but decreasingly; the curve levels off when both proteins have been saturated with calcium. From this point the calcium concentration in the filtrate reaches essentially that of the feeding solution. As this state is reached the UF process may be stopped and the calcium bound to proteins is calculated. However, in routine calcium-binding experiments, the ultrafiltration was left to run until 60 ml of filtrate had been passed through the membrane. By applying eqn (8), the mmoles of calcium bound to the amount of mixed proteins (25 g of whole casein and 3 g of β-lg) present in 1 litre of solution was found to be 6·91. It was reported elsewhere (Pappas, 1979) that the amount of calcium bound to 25 g of whole casein or 3 g of β-lg treated separately at 25°C (without heating) for 30 min was 6·65 or 1·06 mmol, respectively. It is seen that the amount of calcium bound by the mixed protein solution is less than the sum (7·71) of calcium bound by these two proteins when they were treated separately at 25°C. This might be explained by the interaction taking place between whole casein and β-lg at elevated temperatures

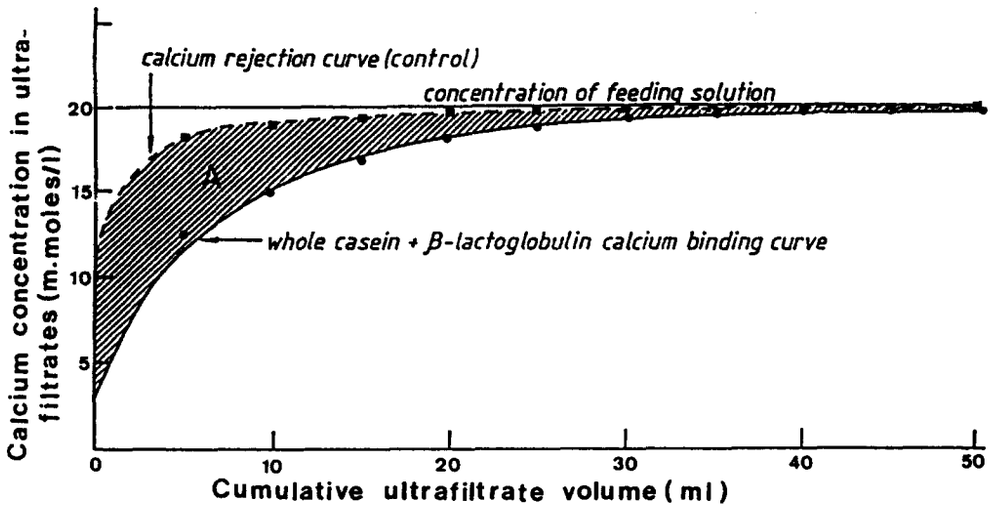

Fig. 4. Calcium rejection of 20 mmol litre^{-1} $CaCl_2$ (control) and calcium binding equilibrium of 2·5% whole casein + 0·3% β-lactoglobulin in 20 mmol litre^{-1} $CaCl_2$, at pH 7·00 and 241·1 kPa (35 psi).

(95°C), which results in the elimination and/or inaccessibility of calcium-binding centres in the molecules of proteins.

The area, A, between the two curves in Fig. 4 represents the amount of calcium bound to the proteins. Yap and Schaffer (1977), studying the binding of bromocresol green to human albumin, by UF, found similar binding patterns to those shown in this study (Figs 4 and 5).

Membrane permeability to milk proteins. The protein retention coefficient, R, and its determination

To examine the permeability of membranes, the protein retention coefficient, R, was determined.

These experiments used both UM10 and PM10 membranes. The 10-ml capacity, stirred, single cell, model 12, was used. β-Lactoglobulin (β-lg) and α-lactalbumin (α-la) were selected for this determination. The discontinuous (batchwise) ultrafiltration technique was applied.

Ten millilitres of 0·3% β-lg or 0·1% α-la were tested with $CaCl_2$ in 3 mmol litre^{-1} sodium barbital buffer, pH 7·00, at 25°C for 30 min, at a final concentration of 10 mmol litre^{-1} with respect to calcium. A pressure of 241·1 kPa (35 psi) was applied. The retention coefficients of the proteins for both membranes were calculated by the formula (Blatt, 1976):

$$R = \frac{\ln (C_f/C_0)}{\ln (V_0/V_f)} \qquad (12)$$

where R is the average retention coefficient of protein by the membrane; C_f is the final protein concentration in retentate, in g litre^{-1}; C_0 is the initial protein concentration in the sample, in g litre^{-1}; V_0 is the initial sample volume, in litres (0·010 litre in this study); and V_f is the final retentate volume, in litres (0·005 litre in this part of the experiments).

The data of Table 1 show that both types of membrane have a very high protein-retention coefficient (> 0·99). Therefore, they were considered suitable for calcium-binding studies, as most of the UF membranes are not ideal in retaining all proteins (Yap & Schaffer, 1977).

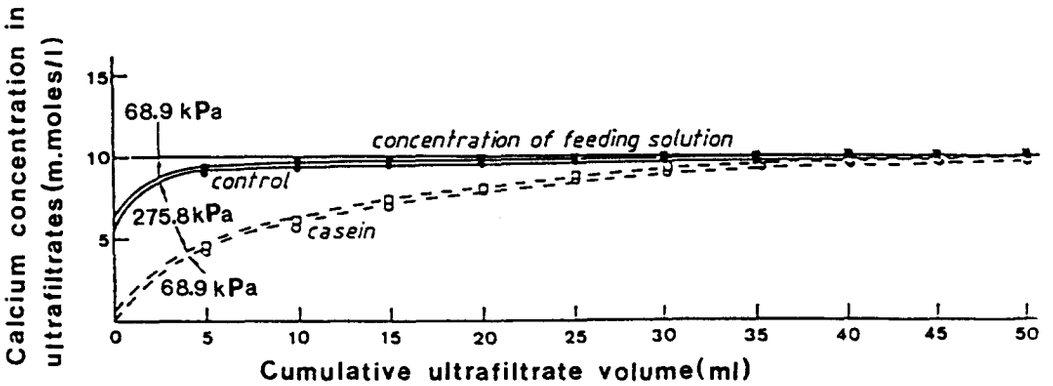

Fig. 5. Continuous ultrafiltration patterns of 10 mmol litre^{-1} $CaCl_2$ (control) and 2·5% whole casein in 10 mmol litre^{-1} $CaCl_2$ at pH 7·00 and 68·9 and 275·8 kPa (10 and 40 psi, respectively).

Table 1. Retention coefficients of milk proteins by different membranes

	Number of 5 ml ultrafiltrates	Absorption at λ =280 nm	Protein concentration in filtrate μg ml^{-1}	Total protein passed through membranes, μg	Total protein losses, %	Retention coefficient R^a
0·3% β-lactoglobulin	1	0·032	16·0	80·0		0·996
Membrane UM 10	2	0·010	0·5	2·5		0·999
	3	0·007	0·4	2·0		0·999
				84·5	0·28	
0·3% β-lactoglobulin	1	0·094	60·0	300·0		0·985
Membrane PM 10	2	0·059	36·0	180·0		0·991
	3	0·028	12·0	60·0		0·997
				540·0	1·80	
0·1% α-lactalbumin	1	0·011	1·5	7·5		0·999
Membrane UM 10	2	0·008	0·7	3·5		0·999
				11·0	0·11	
0·1% α-lactalbumin	1	0·045	14·0	70·0		0·989
Membrane PM 10	2	0·037	11·0	55·0		0·991
	3	0·035	10·5	52·5		0·992
	4	0·025	7·0	35·0		0·995
	5	0·018	4·5	22·5		0·996
				235·0	2·35	

[a] Calculated from eqn (12).

The calcium rejection coefficient and its determination

As it was shown that calcium was rejected by the membrane, the magnitude of the rejection coefficient, σ, was determined. Repeated continuous ultrafiltration experiments without protein, using the multicell ultrafiltration system model MMC and membrane UM10 at 241·1 KPa (35 psi) pressure, were carried out. Four different concentrations of calcium chloride—3, 10, 15 and 20 mmol litre^{-1} in sodium barbital buffer, pH 7·00 —were used, in the presence or absence of lactose. The ultrafiltration cells and reservoir were filled with the same solution. To establish equilibrium conditions, ultrafiltration was extended until 60 ml of ultrafiltrate had been collected. The first 50 ml were discarded; the next 10 ml of ultrafiltrate and the retentate were analysed for calcium. The values of σ, calculated from eqn (3), are given in Table 2. This table indicates that the higher the calcium concentration the lower is the rejection coefficient and that lactose slightly affects σ. When ultrafiltration was allowed to proceed beyond 60 ml of ultrafiltrate to 95 ml, the calcium rejection figure obtained showed a slight decrease, which was considered to be of insufficient significance to justify the extension of ultrafiltration to give 95 ml of ultrafiltrate. The rejection never reaches zero, as the concentration of calcium inside the cell is always higher than in the ultrafiltrates.

The rejection of calcium by the membrane is a complex phenomenon and may be due to the accumulation of calcium on the skin and/or in the pores of the membrane. The presence of ionizable groups attached to the polymer matrix of the membrane could give rise to negatively charged centres, to which ions may be bound. Calcium bound in this way would repel other calcium ions approaching the membrane and thus restrict their passage through the membrane. On the other hand, the fixed charge usually carried by the porous type of membrane excludes ions by the Donnan effect (Glover *et al.*, 1978). Meares (1976) supported the idea that an electrical double layer may be formed on the membrane by ion adsorption, which could be responsible for the rejection of calcium. Ershler (1934) showed that the degree of retention of electrolytes was greater than that of non-electrolytes. The relative concentration

Table 2. Effect of calcium concentration in the presence or absence of lactose on the rejection of calcium by the UF membrane (Membrane UM 10; pressure 241·1 kPa (35 psi); no protein present)

Concentration of feeding solution, mmol litre^{-1}	Concentration of lactose, %	σ^a	Rejection of calcium, %
3	nil	0·121	12·1
3	5	0·147	14·7
10	nil	0·113	11·3
10	5	0·112	11·2
15	nil	0·106	10·6
15	5	0·102	10·2
20	nil	0·087	8·7
20	5	0·089	8·9

[a] Mean values of four experiments.

of filtrate from a solution of non-electrolyte was practically independent of the absolute concentration of the latter; for electrolytes, the relative concentration of filtrate increased markedly with increasing absolute concentration of the original solution. The degree of retention of an electrolyte was greater, the higher the valency of the ion carrying a charge of the same sign as the membrane. This supports the explanation that the greater retention of electrolytes is due to repulsion from the pore walls of similarly charged ions, resulting in a diminution of the effective pore diameter. On the other hand, Ferry (1936) suggested that the rejection of a solute (i.e. calcium) by an ultrafiltration membrane is the result of a 'sieving effect', that is, the partial transmission of a solute, which varies with the porosity of the membrane and the size of the solute particles.

The effect of pressure applied to the ultrafilter on the concentration of calcium in ultrafiltrates and on the amount of calcium bound to protein

It was observed in the previous experiments that the rejection of calcium by the membrane increased when protein was present. This was due to the accumulation of protein on the surface of the membrane, even when the cell contents were well stirred. A protein concentration gradient in the cell liquid is thereby formed, with the highest concentration at the membrane surface, diminishing away from it to the general concentration level in the sample. This phenomenon is referred to as 'the concentration polarization phenomenon' (Blatt, 1976). This polarized layer causes an increase in the rejection of calcium by the membrane and an apparent increase in the amount of calcium bound to protein. Since this phenomenon is affected by the applied pressure, the effects of the pressure on the amount of calcium bound to proteins and on the concentration of calcium in ultrafiltrate were investigated.

Four aliquots of buffered 10 mmol litre^{-1} calcium solution were ultrafiltered and the ultrafiltrates were examined at 5-ml intervals for calcium content; the σ-values were calculated. Four similar experiments were set up using 2·5% whole casein in 10 mmol litre^{-1} buffered calcium chloride solution. Ultrafiltration pressures of 68·9, 103·4, 137·9 and 275·8 kPa (10, 15, 20 and 40 psi, respectively) were applied. The ultrafiltration data of this experiment are summarized in Table 3 and shown in Fig. 5 for the pressures 68·9 and 275·8 kPa (10 and 40 psi, respectively).

The patterns are seen to be almost identical, in spite of the difference in the applied pressure. Table 3 shows that the rejection of calcium by membrane in UF experiments without protein is independent of the operating UF pressure. The concentration of calcium in the ultrafiltrates of casein samples at the beginning of the experiments is always lower than that of the control, because calcium binds to casein. The concentration of calcium in the ultrafiltrates does not change with the applied pressure as can be seen in Table 3, both for the control and the protein samples. However, the amount of calcium bound to casein apparently increases with increasing pressure. This is in agreement with Spector *et al.* (1973), who found an increased apparent binding of digoxin and ouabain to human plasma at higher pressures. By plotting the amount of calcium bound to casein against pressure and extrapolating the lower part of the obtained curve to zero pressure, the true amount of calcium bound to casein was obtained (Spector *et al.*, 1973). The amount of calcium bound to 25 g of casein (6·0 mmol) at zero pressure found in these experiments is about 24% lower than the apparent calcium (7·9 mmol) bound at 275·8 kPa (40 psi). Due to the apparent increase in the amount of calcium bound to protein, caused by high pressures, low operating pressures are preferable in UF-binding studies, a conclusion also reached by Blatt *et al.* (1968).

The value of 24 moles of calcium bound to 10^5 g of whole casein, found in this study using the ultrafiltration technique (Table 3) at zero pressure, is within the values reported in the literature using

Table 3. Influence of pressure applied to ultrafilter on the rejection of calcium and the amount of calcium bound to whole casein

Applied pressure in		Calcium concentration in the retentate, in mmol litre^{-1}		Calcium concentration in ultrafiltrates, in mmol litre^{-1}		σ, control	Calcium bound to	
kPa	(psi)	Control	Whole casein	Control	Whole casein		25 g of whole casein, in mmol[a]	10^5 g of whole casein, in mol[b]
0·0	(0)	—	—	—	—	—	6·0[c]	24·0
68·9	(10)	10·8	17·69	9·96	9·86	0·078	7·0	28·0
103·4	(15)	10·9	18·27	9·96	9·84	0·086	7·5	30·0
137·9	(20)	10·9	18·42	9·93	9·77	0·089	7·7	30·8
275·8	(40)	11·3	19·04	9·94	9·80	0·088	7·9	31·6

[a] Values obtained by applying eqn (8) at an equilibrium-free calcium concentration of 10 mmol litre^{-1}.
[b] Values obtained by applying eqn (11), corrected for σ.
[c] Values obtained by extrapolation to zero pressure.

other techniques. Carr (1953) reported a value of 22 and Zittle *et al.* (1958) a value of 29 moles of calcium per 10^5 g of whole casein.

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