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The Use of Metallic Ultrafiltration Membranes to Assess Calcium Availability *in Vitro*

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ABSTRACT

Three metallic ultrafiltration membranes were studied for potential use in an in-vitro calcium availability testing system. After characterization, the passage of calcium during the enzymatic digestion of casein in the presence or absence of pectin was investigated. The membranes were characterized with respect to passage of calcium and amino acids from solutions of calcium nitrate, amino acids, calcium nitrate and amino acids in combination, casein hydrolysate, and intact casein. The passage of these substances through the membranes was affected by the membrane used and the pH of the solution, as well as by the amino acids present in the solution. It was concluded that this system provided a reasonably efficient and rapid means for obtaining preliminary information about calcium availability.

INTRODUCTION

Several in-vitro techniques involving simulation of gastrointestinal conditions have been devised to investigate mineral availability from selected food material (Hsu *et al.*, 1977; Miller *et al.*, 1981; Gauthier *et al.*, 1982; Zemel, 1984). Most of these procedures involve simulation of gastrointestinal processes accompanied by dialysis (Miller et al., 1981; Gauthier *et al.*, 1982, 1986; Kan & Shipe, 1984; Savoie & Gauthier, 1986). Continual removal of digestion products from the digestion mixture reduces

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the possible inhibitory action of these products and more closely simulates in-vivo processes. For simulation of gastrointestinal tracts, in-vitro 'intestines' of dialysis tubing (Gauthier *et al.*, 1982), semipermeable membranes (Miller *et al.*, 1981), and ultrafiltration (UF) membranes (Camire & Clydesdale, 1981; Kan & Shipe, 1984) have been used. Reactions occurring on the surfaces of these in-vitro 'intestines', however, may alter the passage of digestion products through the membrane. Prior characterization of these membranes, therefore, is necessary to obtain more reliable information about the digestion products released. Thus, the purposes of the present work were, first, to characterize metallic ultrafiltration membranes for possible use in an in-vitro calcium availability system and, secondly, to investigate calcium passage during enzymatic digestion of casein in the presence or absence of pectin.

MATERIALS AND METHODS

Ultrafiltration

The metallic membrane ultrafiltration unit used in these experiments has previously been described by Thomas *et al.* (1986) and was provided by CARRE, Inc. (Seneca, SC). Initially, three different metallic ultrafiltration membranes were studied to determine performance characteristics for in-vitro protein digestibility and calcium availability research. Following characterization, one of these membranes was selected to investigate calcium availability from casein. These membranes were modified zirconium oxide metallic membranes, formed in place on the inside surface of 5/8 in sintered stainless steel tubes. These zirconium oxide membranes were modified to obtain specified characteristics for use in the proposed in-vitro calcium availability system and were denoted UF1, UF2 and UF3. The UF1 and UF2 membranes were designed as lower permeability ultrafiltration membranes than UF3 and they approached reverse osmosis membranes with regard to their permeability. The isoelectric points of the membranes also varied. The UF1 membrane had an isoelectric point of approximately pH 5·0–5·5. The UF2 membrane had an isoelectric point of around pH 7·0–7·5 and the UF3 membrane had an isoelectric point around pH 6·0–7·0. Further information about the formation and composition of these membranes is proprietary. The system included a steam-jacketed kettle which served as both the in-vitro 'stomach' and a temperature-controlled feedtank to maintain the solution at 37°C. Solution was fed into the membrane, the in-vitro 'intestine', at approximately 400 psi.

Characterization

Membranes were characterized with respect to differential passage of calcium and amino acids from solutions of calcium, amino acids, calcium and amino acids in combination, casein hydrolysate, and intact casein. A 1·0% (w/v) solution of casein was run at neutral pH to determine protein and calcium passage characteristics from undigested protein. The membranes were also characterized with respect to passage of free calcium and amino acids. Calcium passage was determined using a 0·02M $Ca(NO_3)_2$ solution at an acidic, neutral, and basic pH for 20 min at each pH. Passage of individual free amino acids was determined using 0·1% (w/v) solutions of various amino acids (Table 1) at an acidic, neutral and basic pH for 20 min at each pH. Analyses were done in duplicate. Based on results of these analyses as

TABLE 1
Passage (%) of Calcium from a 0·02M Calcium Nitrate Solution and Amino Acids from 0·1% (w/v) Solutions

	Membrane		
	UF1	*UF2*	*UF3*
Calcium			
pH 3·4	76	18	91
6·9	88	81	93
8·9	92	94	100
Aspartic acid			
pH 3·8	63	71	89
6·9	36	30	76
8·8	17	28	93
Glycine			
pH 3·8	95	86	97
6·9	100	85	100
8·8	71	67	100
Methionine			
pH 3·8	94	84	97
6·9	92	63	99
8·8	40	32	100
Tyrosine			
pH 3·8	93	80	98
6·9	92	62	100
8·8	37	28	100
Arginine			
pH 3·9	100	87	100
7·0	39	45	100
8·8	60	50	92

TABLE 2
Passage of Amino Acids and Calcium from 1·0% (w/v) Casein Hydrolysate Solution Through the UF3 Membrane

Variable	*Passage* (%)[a]
Aspartic acid	79 ± 1·13[b]
Glutamic acid	79 ± 0·91[b]
Glycine	95 ± 0·94
Methionine	94 ± 1·07
Tyrosine	96 ± 1·00
Arginine	97 ± 1·55
Lysine	98 ± 1·26
Threonine	92 ± 1·54
Serine	95 ± 1·36
Alanine	93 ± 1·56
Valine	93 ± 1·33
Isoleucine	94 ± 1·53
Leucine	95 ± 1·18
Phenylalanine	94 ± 1·31
Histidine	96 ± 0·95
Proline	97 ± 1·92
Calcium	60 ± 2·27

[a] Data are the mean ± standard error of mean from two observations.
[b] Significantly different ($p < 0·01$) from other amino acids.

well as on permeability differences among the membranes, further interactions were studied using only the UF3 membrane.

To determine calcium and amino acid passage in a more complex system, a 1·0% (w/v) casein hydrolysate (Sigma, St Louis, MO) solution was run through the membranes at pH 7·0. Two replicates were performed, with analyses done in duplicate (Table 2). Passage of calcium ion in the presence of charged amino acids was determined using a 0·02M $Ca(NO_3)_2$ solution with acidic, neutral or basic amino acids. These solutions were run separately at an acidic, neutral and basic pH for 30 min at each pH as listed in Table 3.

Digestion procedure

A pepsin digestion followed by a pancreatin–taurocholate digestion was used to determine calcium and amino acid release from casein and

TABLE 3
Passage of Calcium Nitrate and Amino Acids from Combination Solutions through the UF3 Membrane.

Variable	*Passage (%)*		
Acidic amino acids	*3·3*[a]	*6·8*	*8·3*
Aspartic acid	88	61	88
Glutamic acid	92	64	88
Calcium	56	72	89
Neutral amino acids	*3.1*	*7.2*	*8·6*
Methionine	100	91	62
Tyrosine	100	85	83
Calcium	100	93	66
Basic amino acids	*3·4*	*7·2*	*8·6*
Arginine	65	100	88
Lysine	64	100	96
Calcium	83	100	96

[a] Numbers in italic represent pH values.

subsequent passage of these products through the UF3 membrane. For the pepsin digestion, a 1·0% (w/v) solution of casein was adjusted to pH 1·9 with HCl. The casein solution was digested with 2·4 g pepsin (porcine stomach mucosa, 1:10 000) at 37°C for 30 min (Gauthier *et al.*, 1982). This was followed by digestion at pH 7·0 and 37°C for 100–125 min with the following mixture: 2·4 g pancreatin (porcine pancreas, Grade VI), 2·98 g chymotrypsin (bovine pancreas, 40–60 units/mg), and 0·5 g peptidase (porcine intestinal mucosa, 100 units/g) in 240 ml H_2O. Also, 0·1M sodium taurocholate was added to the mixture to a final concentration of 0·01M (Schwartz *et al.*, 1982) and the pH adjusted to 8·0. All enzymes and sodium taurocholate were purchased from Sigma Company (St Louis, MO). The enzyme solution was added in 4 equal increments, at the start of each run and halfway between sample collection times thereafter. Samples were collected 5 min after the run began and every 30 min thereafter. To investigate calcium passage during enzymatic digestion in the presence of fiber, the enzymatic digestion procedure was repeated as previously described except for the addition of 30 g pectin (methoxy content, 7·7%; Sigma Company, St Louis, MO; Camire & Clydesdale, 1981) before initiation of the digestion. Two replicates were performed, with analyses done in duplicate.

Analyses

Amino acid analyses were conducted by the methods of Spackman *et al.* (1958), Moore (1963) and Hugli & Moore (1972), using a Dionex Amino Acid Analyzer (Dionex, Houston, TX). Calcium analyses were done on wet ashed samples (Neidermeir *et al.*, 1971) using atomic absorption spectrophotometry (Hitachi, 1977). The amount of free calcium in casein was determined by first precipitating the protein with 36% (w/v) trichloroacetic acid and then analyzing the amount of calcium in the supernatant using atomic absorption spectrophotometry. Passage through the membranes was determined by comparing the concentration of amino acids and calcium present in the permeate samples with concentration in the feed samples. These values were expressed as percent passage. Pectin analysis was done by the method of Kinter & Van Buren (1982).

Statistical analyses

Analysis of variance and *t*-tests were performed on the data obtained from the casein hydrolysate solution to determine the relative significance of differences in passage among amino acids. The statistical analyses were performed by the Statistical Analysis System (Barr, 1985).

Cleaning

Between runs, the membranes were cleaned with alternating sodium hydroxide (pH 8) and acetic acid (pH 3) washes. Between casein hydrolysate and digestion runs, the membranes were also cleaned overnight with a very dilute protease solution (pH 3) to remove any protein trapped in the matrix of the membrane.

RESULTS

Characterization

With the 1·0% casein solution, no calcium, free amino acids or protein could be detected in the permeate from any of the three membranes. The passage of calcium from the calcium nitrate solution and the passage of amino acids varied with pH and the membrane used (Table 1). Greater variation in calcium and amino acid passage in response to pH changes occurred with the UF1 and UF2 membranes than with the UF3 membrane. At neutral pH, the pH desired for digestion studies, a greater amount of calcium passed

through the UF3 membrane than either the UF1 or UF2 membranes. Aspartic acid was passed less efficiently than the other amino acids at neutral pH, but the UF3 membrane showed greater passage of aspartic acid than either UF1 or UF2. The remaining amino acids were essentially completely passed through the UF3 membrane. Based on results with amino acids and calcium nitrate, the UF3 membrane was selected as the membrane to be used in subsequent calcium availability studies. Consequently, further interactions were studied using only the UF3 membranes.

Only 60% of the calcium from the casein hydrolysate solution passed through the UF3 membrane (Table 2). Since aspartic and glutamic acids were passed less efficiently than other amino acids from the casein hydrolysate solution, interactions occurring between calcium ions and charged amino acids were investigated. In the presence of calcium nitrate, less of the acidic amino acids passed through the membrane compared to either the neutral or basic amino acids (Table 3). Calcium passage with either basic or neutral amino acids was also greater than calcium passage with acidic amino acids present.

During enzymatic digestion, 57% of the calcium present in casein passed through the UF3 membrane. Many of the amino acids were not released in sufficient concentration to accurately determine the percent passage of the amino acids through the membrane.

Reduced passage of calcium was seen when pectin was present during the digestion of casein. When discussing the effect of pectin on calcium passage, however, it is important to consider whether the calcium in pectin was either free initially, released during digestion, or remained bound throughout the digestion procedure, particularly since only 1% of the pectin passed through the membrane. Because of these considerations, the results obtained for calcium passage from casein in the presence of pectin can be presented in two ways. If the calcium from pectin were either free initially or released during digestion and thus contributed to the total pool of free calcium, the source of calcium does not need to be considered in passage calculations because this calcium was available for passage through the membrane. With this assumption, only 21% of the calcium from casein passed through UF3 when pectin was present. If the calcium in pectin remained bound, the calcium contribution from pectin must be subtracted from the total calcium pool before calculating calcium passage. With the assumption that the calcium from pectin remained bound throughout the digestion procedure, calcium passage was 40%. With either assumption, calcium passage during enzymatic digestion of casein in the presence of pectin was less than calcium passage during the digestion of casein alone.

The permeability of the membrane during the digestion of casein alone and in the presence of pectin is presented in Fig. 1. With both digestion

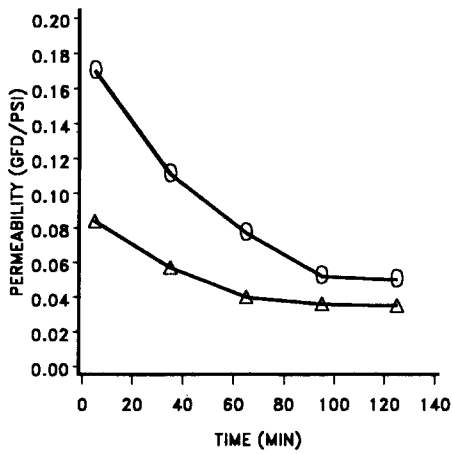

Fig. 1. Permeability (GFD/PSI) of a UF3 membrane during enzymatic digestion of casein in the absence (○) or presence (△) of pectin.

solutions, there was a substantial initial decrease in flux, but after 100 min, the permeability stabilized. As compared to the digestion of casein alone, there was a substantial reduction in flux when pectin was also present. The permeability with the casein digestion averaged 0·082, whereas the permeability in the presence of pectin averaged 0·050.

DISCUSSION

Characterization

The total rejection of calcium from the 1·0% casein solution was expected based on analyses of free calcium in the protein. Although Savoie & Gauthier (1986) found that ionic charge of an amino acid did not influence passage through their dialysis system, the results obtained with the present ultrafiltration membranes indicate that the charge of the membrane, the charge of the components of the solution, and the pH of the solution all influence passage. The net charge and the charge density of the membrane surface vary with pH and with repeated use of the membrane, depending on the type of components binding to the membrane. These charge variations on the membrane affect the passage of charged species. Almost all of the calcium passed through the essentially uncharged UF3 membrane whereas very little calcium passed through UF2, presumably because of the positive charge of both the membrane and the calcium ion. Charge density of the components of the solution may also be important. For example, calcium and arginine are both positively charged throughout the pH range used here.

Calcium, however, was highly rejected at low pH whereas arginine was almost completely passed. Because of the greater passage of calcium and amino acids through the UF3 membrane, the passage of these substances would not be the limiting factor in casein digestion studies.

Enzymatic digestion of casein

The passage of calcium from enzymatically digested casein (57%) was similar to calcium passage from casein hydrolysate (60%) and acidic amino acid/$Ca(NO_3)_2$ combination solutions (72%). It is known that amino acids form stable chelates with metal ions (Sulkowski, 1985); therefore, it is possible that the formation of acidic amino acid–calcium complexes in the casein hydrolysate and in the acidic amino acid/$Ca(NO_3)_2$ combination solutions may decrease passage through membranes due to their size and charge. Acidic amino acid–calcium complexes may have also formed during the digestion of casein, but acidic amino acids were not released in sufficient concentration during the digestion of casein to determine their passage.

The reduced calcium passage from casein in the presence of pectin could be due to one of at least three mechanisms. These are complexation of calcium with pectin, reduction in flux due to membrane fouling, and alteration of charge on the membrane surface, also as a result of fouling. First of all, since low methoxy pectin readily complexes with calcium (Cummings *et al.*, 1979), the reduction in calcium passage may be due to the formation of a calcium–pectate complex too large for passage through the membrane. Secondly, reduced calcium passage could also be due to physical blockage of the membrane pores by the formation of a foulant layer, observed as a decrease in flux. The formation of a foulant layer is substantiated by the different permeability rates observed for the digestion of casein in the presence or absence of pectin and may be important in the lowered passage of calcium (Fig. 1). Finally, if pectin itself became lodged within the pores of the membrane, the carboxyl groups of pectin would alter the charge of the membrane surface and, consequently, the passage of components through the membrane.

With in-vitro systems such as these, careful interpretation of the results is essential. Complexes form *in vivo* which are thought to enhance mineral absorption by increasing the solubility of the mineral and preventing it from forming insoluble complexes with other components of a meal (Allen, 1982; Charley & Saltman, 1963; Hazell, 1985). When these same complexes form *in vitro*, the size and charge of the mineral complex may be such that its passage through membrane systems is prevented. Consequently, the passage of mineral complexes formed *in vitro* may be incorrectly interpreted when applied to humans or animals. For example, in characterization of the

membranes, acidic amino acids and calcium were less diffusable when present together. This does not mean that acidic amino acid–calcium complexes would be unavailable *in vivo*. In fact, acidic species increase solubility and are thus thought to enhance absorption (Wasserman *et al.*, 1956; Charley & Saltman, 1963; Allen, 1982). In biological systems, it is also important to consider that other components of the meal will also influence mineral availability.

Since factors other than molecular weight affect the passage of digestion products through in-vitro 'intestines' such as these, prior characterization of the membranes is necessary to assess more accurately the passage of free mineral and amino acids. This system provided a relatively efficient and rapid means of assessing calcium passage during the enzymatic digestion of casein in the presence or absence of pectin. Again, further investigation into the form in which calcium exists in pectin is required before the validity of the results can be assessed.

Ultrafiltration used in this manner can be used to assess the free mineral present in food material as well as the amount of mineral released during enzymatic digestion. Once a baseline has been established for the mineral of interest, passage patterns can be used to investigate the formation of mineral complexes. An in-vitro system such as the one described here is not a substitute for bioassays, but it does provide useful preliminary information for subsequent animal studies. In-vitro systems are rapid and inexpensive and, in addition, the problem of animal variation, as seen *in vivo*, is eliminated. These systems could provide insight into the formation of complexes which may affect mineral availability—information not as easily obtained from an intact living system.

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