

## Influence of casein phosphopeptides and lactulose on intestinal calcium absorption in adult female rats

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**Summary** — Fractional intestinal Ca absorption was measured in female rats by mixing  $^{47}\text{Ca}$  and the nonabsorbable marker  $^{47}\text{Sc}$  into purified diets and measuring the decrease in the ratio of  $^{47}\text{Ca}$  to  $^{47}\text{Sc}$  in the feces. Ca absorption in 28-wk-old rats consuming a 0.5% Ca diet was not significantly influenced by replacing whey protein with casein, casein phosphopeptides or phosphoserine. Ca absorption was doubled by adding 4% lactulose in both the presence and absence of casein phosphopeptides. Ca absorption in 56-wk-old rats consuming a 0.2% Ca diet was elevated a modest 26% by increasing the level of dietary casein from 9 to 27%.

**absorption / calcium / casein / lactulose / phosphopeptide**

**Résumé** — Effets des phosphopeptides de caséine et du lactulose sur l'absorption intestinale du calcium chez le rat adulte. Une étude sur l'absorption intestinale du calcium chez le rat femelle a été effectuée à l'aide d'une technique de double marquage isotopique au  $^{47}\text{Ca}$  et  $^{47}\text{Sc}$ . La fraction de Ca absorbée d'une diète donnée, a pu être calculée à partir des rapports  $^{47}\text{Ca}$  sur  $^{47}\text{Sc}$  dans ces régimes alimentaires et les selles, le  $^{47}\text{Sc}$  n'étant pas absorbé. Aucun effet significatif sur l'absorption du Ca n'a pu être mis en évidence sur des rats âgés de 28 semaines lorsqu'une partie de la protéine sérique composant la diète est remplacée par de la caséine, des phosphopeptides de caséine ou par de la phosphosérine. En revanche, une adjonction de lactulose à 4% a provoqué une augmentation de 100% de l'absorption du Ca, que les diètes contiennent ou non des phosphopeptides. Chez des rats âgés de 56 semaines, l'absorption du Ca n'augmente que de façon marginale (26%), lorsque la quantité de caséine dans la diète est triplée (9–27%).

**calcium / caséine / absorption / lactulose / phosphopeptide**

### INTRODUCTION

Milk and dairy products are the most important dietary source of Ca in human life and the absorption of Ca from these products is high compared with other foods. The high lactose content of milk is usually considered to be a major factor responsible for the high Ca absorption from milk and dairy products. As reviewed by Miller (1989), lactose has repeatedly been

shown to stimulate intestinal Ca absorption in rats. This beneficial effect of lactose was also observed in infants receiving a soy protein-based formula with either lactose or corn syrup as the carbohydrate source (Ziegler and Fomon, 1983). A cow's milk-based infant formula was shown to give higher bone mineralization in infants in comparison with a soy-based, lactose-free formula (Steichen and Tsang, 1987). In adult humans the effect

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of lactose on Ca absorption is still a matter of controversy (Miller, 1989).

In addition to lactose, casein has been suggested to further enhance Ca absorbability. The phosphorylated regions of the various bovine caseins are involved in stabilizing the colloidal Ca phosphate complex of cow's milk (Holt and Sawyer, 1988). Casein derived phosphopeptides bind metal ions (Oesterberg, 1966) and stabilize supersaturated Ca phosphate systems (Reeves and Latour, 1958; Gerber and Jost, 1986; Berrocal *et al*, 1989).

*In vivo* digestion of casein produces phosphopeptides which enhance the intestinal solubility of Ca according to Naito *et al* (1972). The amounts of soluble Ca and P in rat intestine were higher after a meal with casein than other dietary proteins (Lee *et al*, 1980). The *in vivo* formation of a defined phosphopeptide was recently demonstrated in cannulated minipigs fed casein, and the casein fragment  $\alpha_{s1}$  [66–74] isolated by preparative HPLC (Meisel and Frister, 1988). These observations are compatible with a role of casein phosphopeptides on the intestinal solubility and absorption of Ca but they provide no direct evidence for a stimulation of Ca absorption.

A balance study with young pigs fed a 0.8% Ca diet for 52 d showed no influence of 5% casein phosphopeptides on intestinal Ca absorption (Pointillart and Guégen, 1989). Despite the absence of a long-term effect, a transient stimulation of Ca absorption might have occurred. Thus, a short-term assay might be a more sensitive test of the ability of casein phosphopeptides to enhance Ca absorption. This approach was chosen in our study in which intestinal Ca absorption from  $\text{CaCO}_3$  was compared in rats fed diets containing whey protein, casein, casein phosphopeptides or phosphoserine as nitrogen sources.

## MATERIALS AND METHODS

### Materials

Lactulose and phosphoserine were purchased from Fluka, Switzerland. The whey protein isolate (BiPRO) was purchased from Bio-Isolates, UK and vitamin-free casein was purchased from ICN Biomedicals, USA. Casein phosphopeptides were produced as already described (Juillerat *et al*, 1989) by tryptic hydrolysis of whole bovine casein, followed by separation of the pH 4.5 soluble peptides on an anion exchange column. Protein values were calculated from 6.38 times their nitrogen contents. Further details on these proteins are given in table 1.  $^{47}\text{Ca}$  and  $^3\text{H}$ poly-ethyleneglycol were purchased from Amersham, England.

### *In vitro* stability of phosphopeptides

The small intestine of an adult rat was dissected into 3 segments corresponding approximately to duodenum, jejunum and ileum. Each segment, including its contents, was immediately extracted with 5 ml of ice-cold 0.02 M pH 8.0 Tris-HCl buffer. Following removal of insoluble material by centrifugation, phosphatase activity was measured and found highest in the ileum. Casein phosphopeptides (10 mg/ml) were incubated with the Tris-buffered ileal extract for up to 3 h at 25 °C. Samples were chromatographed on a Mono-Q anion exchange column (Juillerat *et al*, 1989) and, from the decrease in the respective peak areas, the rate of dephosphorylation of the phosphopeptides was calculated. In order to measure the liberation of inorganic P from the peptides, an aliquot of the incubation mixture was treated with 2 N  $\text{H}_2\text{SO}_4$  and assayed for inorganic P according to the procedure of Van Veldhoven and Mannaerts (1987).

### *In vivo* stability of phosphopeptides

1.5 ml of a 40 mg/ml casein phosphopeptide solution in 0.1 M  $\text{CaCl}_2$  was administered together with 2  $\mu\text{Ci}$  of  $^3\text{H}$ polyethyleneglycol by stomach gavage to a 250-g rat. One h later the

**Table I.** Ca and P contents of dietary components. Protein contents were calculated from 6.38 times the nitrogen contents. ND indicates that no measurement was performed.

*Composition des éléments constituant le régime alimentaire. Un facteur de conversions de 6,38 a été utilisé pour calculer la quantité de protéines à partir de la teneur en azote. ND = non déterminé.*

Component	Protein (% weight)	Calcium (mg/100 g)	Total P (mg/100 g)	Inorganic P (% total P)	Ash (%)
Casein	90.0	45.4	775	0.8	1.1
Whey protein	93.4	161.9	25	34.8	1.5
Casein phosphopeptides	76.6	50.0	3 020	0.1	12.5
Phosphoserine	48.3	0	16 736	0.1	ND
Cornstarch	0.1	13.7	12	ND	0.1
Cellulose	0	74.5	0	0	0.1

rat was sacrificed and the small intestine divided into the 3 segments described above which were then extracted with ice-cold pH 7.0 Tris buffer. The extracts were centrifuged and supernatants assayed for radioactivity and chromatographed on the Mono-Q column.

### Intestinal Ca absorption

Female rats of the Fischer 344 strain were obtained from IFFA-CREDO (Les Oncins, France) at 15 wk of age. Intestinal Ca absorption from diets consumed during a single night was measured during wk 28 and 56 of age. For the first study examining the effect of casein phosphopeptides, the rats were fed a pelleted diet containing 0.53% Ca as CaCO<sub>3</sub>, 0.35% P, 5% corn oil and 11.2% whey protein for 3 wk preceding the experiment. For the second study examining the influence of different levels of dietary casein, the rats were fed a pelleted diet containing 0.2% Ca as CaCO<sub>3</sub>, 0.5% P, 7% corn oil and 18% casein for 3 wk preceding the test. In both studies, the diets contained 15% sucrose, 3% cellulose, the AIN-76A concentrations of DL-methionine (0.2%), choline bitartrate (0.3%), vitamins and minerals (except Ca and P), with corn starch as the major carbohydrate source.

Fractional Ca absorption was determined from the decrease in the ratio of <sup>47</sup>Ca to <sup>47</sup>Sc in feces relative to diet using the method of McCredie *et al* (1984) modified to permit inclusion of <sup>47</sup>Ca in the diet rather than giving the isotope by

stomach gavage. The gamma-emitting <sup>47</sup>Ca (*t*<sub>1/2</sub> = 4.53 d; energies of 490, 810 and 1 290 keV) decays to the gamma-emitting <sup>47</sup>Sc (*t*<sub>1/2</sub> = 3.43 d; energy of 160 keV). Both isotopes were counted simultaneously by discrimination of their gamma energies. Since <sup>47</sup>Sc is not absorbed by the intestine, it serves as a nonabsorbable marker and thus only representative samples of both diet and feces need to be counted.

The test diets were identical to the adaptation diets except for the specific changes described in the legends to figures 3 and 4 and the addition of the radioactive tracers and food coloring. <sup>47</sup>Ca (50 µCi) and <sup>47</sup>Sc (≈ 30 µCi) were added to a powdered diet containing 0.01% Sc as ScCl<sub>3</sub> (a carrier for the <sup>47</sup>Sc) and the food dye Fast Green FCF (0.03%). The dye permitted easy identification of the radioactive feces. The diets were thoroughly mixed using a kit-chen-type blender and water was added to produce a thick paste that could be formed into pellets by hand. The formation of these pellets was facilitated by using cornstarch as the major carbohydrate source.

On the day of each experiment, the adaptation diet was removed during the morning and replaced with the radiolabeled diet in the evening just prior to darkness. The radiolabeled diet was removed the following morning and replaced with the normal diet. An advantage of this assay is that the rats consume the test diets under normal feeding conditions. Feces were collected during the following 4 d with care taken to count only the green-colored radioac-

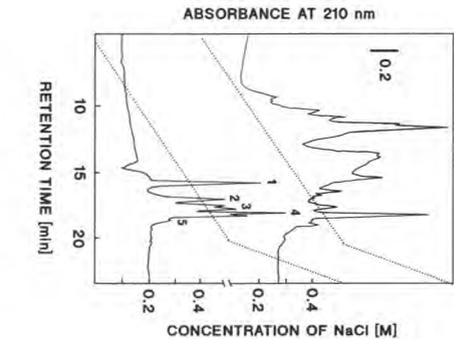
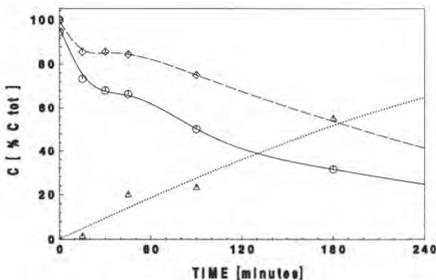
tive feces. Throughout the experiment the rats remained in their individual plastic cages with wood chips as bedding material. Demineralized water was provided during the experiment.

A Packard Auto-Gamma counter was employed with the pre-set window for  $^{57}\text{Co}/^{75}\text{Se}$  used to detect  $^{47}\text{Sc}$  and an energy range of 360 to 1 560 keV for  $^{47}\text{Ca}$ . Diet and fecal samples were counted in plastic tubes (11 mm internal diameter and 75 mm height) with care taken to fill each tube to a height of 56 mm in order to maintain counting efficiencies constant. The sample depth control was set to 2.5 cm. Appropriate corrections were made for the 21% spill-over of  $^{47}\text{Ca}$  counts into the  $^{47}\text{Sc}$  channel. From the ratios of  $^{47}\text{Ca}/^{47}\text{Sc}$  in diets and feces, fractional Ca absorption was calculated as described by McCredie *et al* (1984).

## RESULTS

### *In vitro* and *in vivo* stabilities of casein phosphopeptides

During *in vitro* incubation of the casein phosphopeptides with extracts from rat ileum, there was a progressive rise in inorganic P which reached a level of  $\approx 50\%$  of the total initial organic P content of the peptides after 3 h (fig 1). Chromatographic analysis showed the cumulated decrease

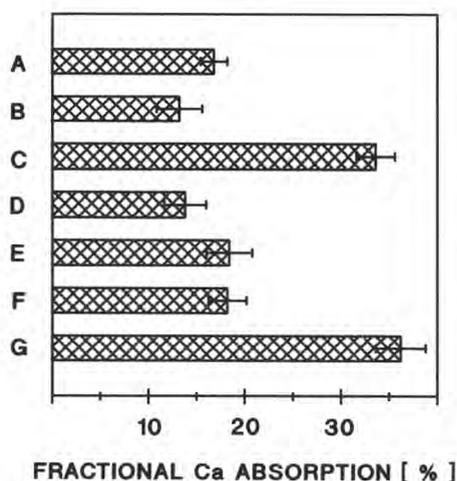


**Fig 2.** *In vivo* stability of casein phosphopeptides. FPLC profiles of the phosphopeptide preparation given to the rats (lower profile) and the ileal extract 1 h after stomach gavage (upper profile). The phosphopeptides are identified as follows:  $\beta$ -cas [33-48];  $\alpha_{s1}$ -cas [43-58];  $\beta$ -cas [1-28];  $\alpha_{s1}$ -cas [59-79] and  $\beta$ -cas [1-25]; and  $\alpha_{s2}$ -cas [46-70] for peaks 1 through 5, respectively.

*Stabilité in vitro des phosphopeptides de caséine. Le chromatogramme (FPLC) du bas représente la fraction de phosphopeptides administrée aux rats et le profil du haut représente l'extrait de l'iléum 1 h après gavage. Les peptides suivants ont été identifiés: 1)  $\beta$ -cas [33-48]; 2)  $\alpha_{s1}$ -cas [43-58]; 3)  $\beta$ -cas [1-28]; 4)  $\alpha_{s1}$ -cas [59-79] et  $\beta$ -cas [1-25]; 5)  $\alpha_{s2}$ -cas [46-70].*

**Fig 1.** *In vitro* dephosphorylation of casein phosphopeptides by rat ileal extracts. Samples were analyzed by FPLC chromatography using a Mono-Q resin as described by Juillerat *et al* (1989). All data are presented as percentages of initial concentrations. The solid line represents the total of all phosphopeptide peaks, the dashed line the  $\alpha_{s1}$  [59-79] fragment, and the dotted line the release of inorganic P.

*Déphosphorylation in vitro de phosphopeptides de caséine par des extraits d'iléum de rat. Les échantillons ont été analysés par FPLC selon Juillerat et al (1989). L'ordonnée représente le pourcentage de la concentration initiale. Les phosphopeptides totaux sont représentés par un trait plein, le fragment  $\alpha_{s1}$  [59-79] par une ligne fragmentée et le P inorganique par un trait pointillé.*



**Fig 3.** Intestinal Ca absorption in rats fed diets containing 0.53% Ca and 0.35% P. There were 8 rats in each group and data are presented as means  $\pm$  SEM. The groups were (A) 11.2% whey protein; (B) 11.2% casein; (C) 11.2% whey protein and 4% lactulose; (D) 10.3% whey protein and 1.8% phosphoserine; (E) 7.4% whey protein and 3.8% phosphopeptides; (F) 3.6% whey protein and 7.6% phosphopeptides; and (G) 3.6% whey protein, 7.6% phosphopeptides and 4% lactulose. Each diet contained 11.2% total protein.  $\text{CaCl}_2$  was used to correct for the small differences in endogenous Ca contents of each diet. Dietary P was provided by the endogenous P of the protein sources and  $\text{KH}_2\text{PO}_4$ . Phosphoserine was added to provide an equivalent quantity of organic P as the 7.6% phosphopeptide group and lactulose replaced an equivalent quantity of cornstarch. An analysis of variance ( $F$  value = 0.21;  $P > 0.2$ ) demonstrated that there were no significant differences in Ca absorption among groups A, B, D, E and F. Ca absorption was elevated in both groups receiving lactulose at  $P < 0.01$  as these values are outside the 99% confidence limits of the other 5 groups.

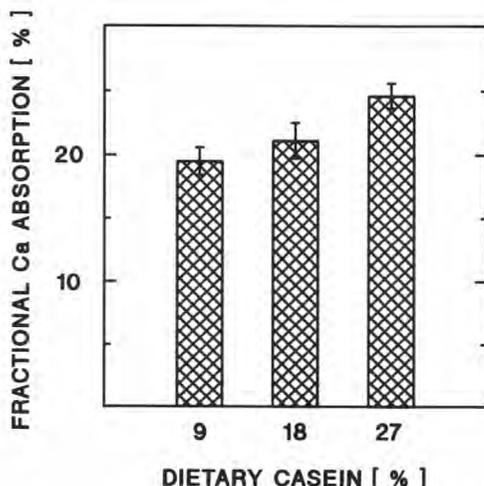
*Absorption du Ca chez le rat soumis à des diètes contenant 0,53% Ca et 0,35% P. Chaque groupe est composé de 8 rats et les valeurs moyennes  $\pm$  SEM sont représentées dans la figure. Les diètes sont composées de : (A) 11,2% de protéines de sérum laitier; (B) 11,2% de caséine; (C) 11,2% de protéines sériques et 4% de lactulose; (D) 10,3% de protéines sériques et 1,8% de phosphosérine; (E) 7,4% de protéines sériques et 3,8% de phosphopeptides; (F) 3,6% de protéines sériques et 7,6% de phosphopeptides; (G) 3,6% de protéines sériques et 4% de lactulose. Le taux de protéine totale a été maintenu à 11,2%. La concentration des diètes en Ca et en P a été maintenue à niveau constant par l'adjonction de  $\text{CaCl}_2$  et  $\text{KH}_2\text{PO}_4$ . La phosphosérine a été ajoutée en quantité correspondante à la quantité de phosphore présent dans 7,6% de phosphopeptides. Dans les diètes contenant du lactulose, ce dernier remplace en quantité équivalente l'amidon de maïs. Aucune différence significative n'a pu être mise en évidence entre les groupes A, B, D, E et F en utilisant une analyse de variance ( $F = 0,21$ ;  $P > 0,2$ ). Seules les diètes contenant du lactulose ont provoqué une augmentation significative de l'absorption de Ca.*

in the peak areas corresponding to all phosphopeptides. The highest rate of dephosphorylation was observed during the first 15 min of incubation. One of the casein fragments,  $\alpha\text{s}_1$  [59–79], was more slowly dephosphorylated than the other fragments.

One h after administration of the peptide–polyethyleneglycol mixture by stomach gavage, the main fraction of radioactivity was recovered in the ileum. Extracts of all 3 segments of the small intestine showed the duodenal and jejunal segments to be essentially devoid of phosphopeptides, while phosphopeptides were found in the ileum. Chromatographic analysis (fig 2) showed that the fragment  $\alpha\text{s}_1$  [59–79] was the most abundant phosphopeptide present. This observation is interesting in view of the presence of a similar fragment,  $\alpha\text{s}_1$  [66–74], found by Meisel and Frister (1988) in minipigs fed a casein meal.

### Intestinal Ca absorption

Ca absorption from a 0.5% Ca diet as influenced by different proteins and lactulose is showed in figure 3. Ca absorption from a whey protein-based diet was not significantly different from that of a casein-based diet nor from diets in which part of the whey protein had been replaced by casein, casein phosphopeptides or phosphoserine, while keeping the total protein



**Fig 4.** Fractional intestinal Ca absorption in rats fed diets containing different levels of casein. There were 13 rats in each group and data are presented as means  $\pm$  SEM. Due to the endogenous Ca and P contents of the casein, dietary Ca and P levels varied from 0.20 to 0.21% and 0.42 to 0.57%, respectively. An analysis of variance gave a *F* value of 4.88 indicating a significant difference among the 3 groups at  $P < 0.01$ . *Fraction du Ca absorbé chez le rat à partir d'un régime alimentaire contenant des quantités croissantes de caséines. Chaque groupe est composé de 13 rats. Les valeurs moyennes  $\pm$  SEM pour chaque groupe sont représentées ci-dessus. La concentration du Ca et P fluctuent respectivement entre 0,20–0,21% et 0,42–0,57%. Ces variations sont dues au Ca et P endogène de la préparation de caséine utilisée. Une analyse de la variance ( $F = 4,88$ ) montre une différence significative entre ces 3 groupes ( $P < 0,01$ ).*

level of the diets constant at 11.2%. In contrast, addition of 4% lactulose doubled Ca absorption. This strong enhancement of Ca absorption by lactulose was not affected by the presence of the casein phosphopeptides. Ca absorption from a 0.2% Ca diet was slightly enhanced by increasing the casein content of the diet from 9 to 27% (fig 4).

### DISCUSSION

Several authors have suggested that casein phosphopeptides enhance intestinal Ca absorption. However, Pointillart and Guégen (1989) found no evidence for such an enhancement in young pigs fed chymotryptic casein phosphopeptides for 52 days. In addition, Li *et al* (1989) could find no evidence for a direct stimulatory effect of casein phosphopeptides on intestinal Ca transport in the rat ileum as these peptides inhibited the mucosal to serosal transfer of Ca in direct relationship to the reduction in mucosal ionized Ca concentrations.

In the present study, intestinal Ca absorption in adult female rats was measured over a period of one night while the rats maintained their normal eating patterns. Our assay readily detected the stimulatory effect of lactulose, and in other experiments (data not shown), the inhibiting effects of phytate and oxalate. Our failure to find any significant stimulation of Ca absorption indicates that phosphopeptides do not play a decisive role in the absorption process. This lack of stimulatory activity cannot be explained by an excessively rapid dephosphorylation or hydrolysis in the intestine as we have provided chromatographic evidence for the presence of at least part of the phosphorylated species in the ileum 1 h after stomach gavage.

The mechanism by which increasing levels of dietary casein enhance Ca ab-

sorption is unclear but unlikely to be related to the formation of phosphopeptides since tripling the dietary casein level resulted in only a minor elevation of Ca absorption. This modest enhancement of Ca absorption (26%) is not specific for casein as it is also observed with soy protein (R Brommage, unpublished results).

In light of these recent studies, a role for casein phosphopeptides in intestinal Ca absorption appears to be questionable.

The mechanism by which lactulose stimulates intestinal Ca absorption is unclear but this analogue of lactose may act in a similar manner to lactose. However, in contrast to lactose, lactulose is not metabolized in the small intestine and thus may be effective at lower dietary levels than lactose. Further experiments are underway to understand the mechanism of the stimulation of intestinal Ca absorption by lactulose.

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## REFERENCES

- Berrocal R, Chanton S, Juillerat MA, Pavillard B, Scherz JC, Jost R (1989) Tryptic phosphopeptides from whole casein. II. Physicochemical properties related to be solubilization of calcium. *J Dairy Res* 56, 335-341
- Gerber HW, Jost R (1986) Casein phosphopeptides: Their effect on calcification of *in vitro* cultured embryonic rat bone. *Calcif Tissue Int* 38, 350-357
- Holt C, Sawyer L (1988) Primary and predicted secondary structures of the caseins in relation to their biological function. *Protein Eng* 2, 251-259
- Juillerat MA, Baechler R, Berrocal R, Chanton S, Scherz JC, Jost R (1989) Tryptic phosphopeptides from whole casein. I. Preparation and analysis by fast protein liquid chromatography. *J Dairy Res* 56, 603-611
- Lee YS, Noruchi T, Naito H (1980) Phosphopeptides and soluble calcium in the small intestine of rats given a casein diet. *Br J Nutr* 43, 457-467
- Li Y, Tomé D, Desjeux JF (1989) Indirect effect of casein phosphopeptides on calcium absorption in rat ileum *in vitro*. *Reprod Nutr Dev* 29, 227-233
- McCredie DA, Troehler U, Bonjour JP (1984) *In vivo* determination of intestinal calcium absorption, with scandium 47 used as marker. *J Lab Clin Med* 103, 354-362
- Meisel H, Frister H (1988) Chemical characterization of a caseino-phosphopeptide isolated from *in vivo* digests of a casein diet. *Biol Chem Hoppe-Seyler* 369, 1275-1279
- Miller DD (1989) Calcium in the diet: food sources, recommended intakes, and nutritional bioavailability. *Adv Food Nutr Res* 33, 103-154
- Naito H, Kawami A, Imamura T (1972) *In vivo* formation of phosphopeptide with calcium-binding property in the small intestinal tract of the rat fed on casein. *Agric Biol Chem* 36, 409-415
- Oesterberg R (1966) Phosphorylated peptides, study of their primary structure and metal complexity. Ph D Thesis, Uppsala, Sweden
- Pointillart A, Guégen L (1989) Absence d'effet de l'incorporation d'un phosphopeptide du lait sur l'utilisation du calcium et du phosphore chez le jeune porc. *Reprod Nutr Dev* 29, 477-486
- Reeves RE, Latour NG (1958) Calcium sequestering phosphopeptide from casein. *Science* 128, 472
- Steichen J, Tsang RC (1987) Bone mineralization and growth in term infants fed soy-based or cow milk-based formula. *J Pediatr* 110, 687-692
- Van Veldhoven PP, Mannaerts GP (1987) Inorganic and organic phosphate measurements in the nanomolar range. *Anal Biochem* 161, 45-48
- Ziegler EE, Fomon SJ (1983) Lactose enhances mineral absorption in infancy. *J Pediatr Gastroenterol Nutr* 2, 288-294