Calcium absorption and bone utilization in spontaneously hypertensive rats fed on native and heat-damaged casein and soya-bean protein

BY YVONNE V. YUAN AND DAVID D. KITTS*

Department of Food Science, Faculty of Agricultural Sciences, University of British Columbia, 6650 N.W. Marine Drive, Vancouver, B.C. V6T 1Z4, Canada

(*Received 12 September 1992 – Revised 16 June 1993 – Accepted 8 July 1993*)

The effects of dietary protein on Ca bioavailability and utilization in bone were examined in male spontaneously hypertensive rats (SHR) fed on diets containing either casein (200 g/kg (control), 60 g/kg or heat-damaged (HD) 200 g/kg) or soya-bean protein isolate (200 g/kg (control), 60 g/kg, or HD 200 g/kg). Casein was heat-damaged to limit caseinophosphopeptide (CPP) production in order to evaluate casein enhancement of Ca bioavailability. All diets contained an adequate level of Ca (5 g/kg). A 24 h mineral balance study was performed when animals were 10 weeks old, followed by measurement of *in situ* paracellular Ca disappearance, femur mineralization and biomechanics at 14 weeks of age. Digestibility of soya-bean and both HD proteins estimated *in vitro* was reduced compared with native casein. Animals fed on HD and 60 g/kg protein diets exhibited decreased ($P < 0.05$) body weight gain, dry matter intake and feed efficiency compared with controls. The ileal disappearance of ^{45}Ca was lower ($P < 0.05$) in animals fed on HD casein and all the soya-bean protein diets. Ca balance was not strongly affected by dietary treatments. A significant ($P < 0.05$) interaction between protein source and reduced protein intake was observed for femur calcification and physical measurements. Femur bending failure energy and biomechanical force measurements were reduced ($P < 0.05$) in HD and 60 g/kg casein and soya-bean protein fed animals. These findings suggest that whole-body Ca homeostatic mechanisms were involved in compensating for reduced Ca bioavailability and retention from casein diets modified to reduce protein digestibility and CPP production.

Caseinophosphopeptides: Calcium: Femur: Protein: SHR

The high incidence of osteoporosis among the aged has resulted in an increased focus on dietary sources of Ca and the controversial use of Ca supplements for controlling age-related bone loss (Heaney *et al.* 1982; Dawson-Hughes *et al.* 1987). Previous reports have indicated that dairy products are excellent sources of available Ca compared with cereal-legume foods (Liebman & Landis, 1989). Various constituents of milk, namely the carbohydrate lactose (Sato *et al.* 1983*a*; Shortt & Flynn, 1991; Yuan *et al.* 1991) and casein tryptic digestion products (caseinophosphopeptides (CPP) Naito *et al.* 1972; Lee *et al.* 1980; Nagasawa *et al.* 1991) have been reported to enhance Ca absorption in the ileum by facilitating paracellular absorption.

CPP have been shown to prevent the formation of insoluble Ca-phosphate precipitates in the intestinal milieu by complexing ionized Ca in soluble chelates which enhance the amount of intraluminal Ca available for transport across the mucosa by the non-saturable pathway (Naito *et al.* 1972; Naito & Suzuki, 1974). However, studies feeding a synthetic phosphopeptide ($(Ser\text{-}P)_3$ Glu) added to soya-bean protein diets were unable to show an

* For reprints.

effect on apparent absorption of Ca or femoral Ca deposition (Shah *et al.* 1990). More recent experiments have shown that CPP supplementation of soya-bean diets enhanced intestinal Ca solubility and ileal paracellular absorption *in situ*, but had little effect on Ca balance and utilization in bone calcification when diets contained adequate Ca (Yuan & Kitts, 1991). Other workers have also indicated that increasing the level of casein in the diet three-fold resulted in an enhanced Ca absorption (Brommage *et al.* 1991). This result was not unique to casein, however, thereby suggesting that this effect was not specifically related to CPP production. In both spontaneously hypertensive (SHR) and normotensive rats, femoral mineralization and biomechanical strength variables have been shown to be influenced primarily by the level of dietary Ca intake (Yuan & Kitts, 1992*a*), and not so much by casein or soya-bean protein sources (Yuan & Kitts, 1992*b*). Thus the physiological significance of CPP-mediated increased paracellular Ca absorption when dietary Ca levels are normal remains equivocal.

Although CPP fragments have been identified in the intestinal contents of animals fed on casein (Naito *et al.* 1972; Meisel & Frister, 1989), the production of CPP digestion peptides may be affected by factors influencing proteolytic activity and intestinal secretion (Lee *et al.* 1980; Yvon & Pelissier, 1987). Moreover, the digestibility of dietary proteins can influence pancreatic enzyme composition and turnover (Percival & Schneeman, 1978). In the present study two approaches for evaluating the potential effect of CPP on Ca absorption were made, first by altering the potential production of CPP through the reduction of dietary protein intake, and second by drastically reducing protein digestibility. Since macropeptides derived from soya-bean proteins have limited capacity for enhancing Ca solubility (Sato *et al.* 1986; Kitts *et al.* 1992), this protein source was used as a reference protein for evaluating CPP activity on Ca bioavailability and utilization. Ca bioavailability estimates were made from both *in situ* and balance studies. Specific biomechanical tests for bone strength (Yuan & Kitts, 1992*b*) were used as additional indices of Ca utilization. The SHR model was used because of its susceptibility to the development of osteoporotic bone disorders (Izawa *et al.* 1985).

MATERIALS AND METHODS

Heat treatment of proteins

Casein and soya-bean protein isolate (ICN Biochemicals Inc., Cleveland, OH, USA) were heat-damaged (HD) according to the method of Percival & Schneeman (1979), by autoclaving the proteins at a sterilization temperature of 121°, 2 atmospheres for 24 h, followed by cooling to room temperature.

In vitro *digestibility study*

A two-step proteolysis method using pepsin (*EC* 3.4.23.1) followed by pancreatin enzymic hydrolysis was used in a modification of the protocol of Jacques *et al.* (1986). Proteins, namely native casein, soya-bean protein isolate, HD casein and HD soya-bean protein were individually suspended in deionized water. Suspensions were adjusted to pH 1·9 with dilute HCl, and incubated with pepsin (porcine stomach mucosa, 1:10000) in a shaking water bath (37°, 30 min). The samples were adjusted to pH 8 using $NaHCO_3$ and they were reincubated with pancreatin (porcine pancreas; Grade II; Sigma, St. Louis, MO, USA) at 37°. Portions were removed at frequent intervals for deproteinization with trichloroacetic acid (TCA; 200 g/l) and then reacted with 2,4,6-trinitrobenzenesulphonic acid (Eastman Kodak, Rochester, NY, USA) according to the method of Kwan *et al.* (1983). The initial slopes of protein digestion following the two-step enzymic digestion

treatments were obtained by linear regression analysis (Maga *et al.* 1973), for comparison between the initial proteolysis rates of native and HD proteins.

In vivo *study*

Thirty-six male SHR (4 weeks old; Charles River, Montreal, Quebec, Canada) were divided into six dietary groups (six animals per group). Animals were individually housed in stainless steel cages in a room with controlled temperature (25°) and lighting (14 h light–10 h dark cycle). The six dietary treatments were 200 g casein/kg, 200 g soya-bean protein isolate/kg, 60 g casein/kg, 60 g soya-bean protein isolate/kg and 200 g HD casein/kg and 200 g HD soya-bean protein isolate/kg (Table 1). All diets contained an adequate level of Ca (5 g/kg), and contained 2 g polyethylene glycol (PEG)/kg as a non-absorbable marker.

Animals were fed *ad lib.* until they reached 100 g body weight, after which a meal-feeding protocol was initiated. Animals were trained to consume the respective diets within a 6 h period daily (09·00 hours to 15·00 hours) over a 2 week training period. Deionized water was available *ad lib.* After the meal-feeding training period, daily feed intakes and weekly body weight gains were recorded throughout the experiment. Animals were cared for in accordance with the principles of the *Guide to the Care and Use of Experimental Animals* of the Canadian Council on Animal Care (1984).

Mineral balance study. A 24 h balance study was performed when animals were 10 weeks of age. Animals from each dietary group (*n* 6) were placed in individual metabolic cages for acclimatization 24 h before sample collection. Sample collections were made for a further 24 h after acclimatization. Metabolic cages (Nalgene, Rochester, NY, USA) for 150–300 g rats were equipped with collection funnels and separation cones to separate urine and faeces, and to eliminate urine washover and contamination of faeces. Separation of excreta was immediate and complete using this apparatus. Urine and faecal samples were collected, weighed, and frozen until analysis. Feed intake and water consumption were also measured during the 24 h balance study period. Ca and Mg were determined by atomic absorption spectrophotometry in the presence of $LaCl_3$ (5 g/l). Urinary P was measured by the colorimetric method of Itaya & Ui (1966).

In situ *calcium absorption.* Ca absorption was measured using an *in situ* ligated ileum technique in meal-fed animals (14 weeks of age), as previously reported (Lee *et al.* 1980; Nagasawa *et al.* 1991). On the morning of the experiment rats were allowed access to their respective diets for a 1·5 h period. Rats were anaesthetized with a vapour mixture of halothane and O_2 (4% halothane at a flow rate of 4 l/min for induction, and 2·5% at a flow rate of 2 l/min to maintain anaesthesia during the surgical procedure), and intestinal surgery commenced 1·5 h after removal of the diets. The small intestine was exposed and the ileum isolated, keeping the intestinal contents intact, between two ligatures placed at points 80 and 200 mm from the ileocaecal junction. A dose (300 μl) of ^{45}Ca (0·2 MBq $^{45}CaCl_2$, S.A. 0·674 GBq/mg Ca; ICN Biomedical, Irvine, CA, USA) in 0·15 M-NaCl was injected into the closed sac. Care was taken to massage the ligated ileal segment gently to obtain uniform distribution of the radioactivity in the loop contents. The absorption of ^{45}Ca was determined by calculating the percentage of dose absorbed, from the amount of original dose remaining in the ligated ileal segment after a 1 h period (Sato *et al.* 1983*a*; Nagasawa *et al.* 1991). ^{45}Ca was measured with an LKB-1215 liquid scintillation counter (Wallac Oy, Turku, Finland). The ^{40}Ca in the ileal contents was measured by atomic absorption spectrophotometry (Perkin Elmer-306 atomic absorption spectrophotometer; Perkin Elmer, Norwalk, CT, USA) following wet ashing with HCl-HNO_3 (Mauer, 1977) and dilution with $LaCl_3$ (5 g/l). Intestinal sac contents samples were deproteinized with TCA and centrifuged (10000 ***g***) for 20 min to recover TCA-soluble material. TCA was

Table 1. *Composition of diets (g/kg) fed to experimental animals*

Dietary component	Casein protein diets			Soya-bean-protein diets		
	Heat damaged	60 g protein/kg	200 g protein/kg (control)	Heat damaged	60 g protein/kg	200 g protein/kg (control)
Casein*	200	60	200	—	—	—
Soya-bean-protein isolate*	—	—	—	200	60	200
DL-methionine†	3	3	3	3	3	3
Maize starch*	150	150	150	150	150	150
Sucrose	487·7	600	487·7	487·7	600	487·7
Fibre*	50	50	50	50	50	50
Vegetable oil	50	50	50	50	50	50
Ca-free mineral mix*	35	35	35	35	35	35
Vitamin mixture*	10	10	10	10	10	10
Choline bitartrate†	2	2	2	2	2	2
Calcium carbonate‡	11·7	11·7	11·7	11·7	11·7	11·7
Polyethylene glycol	20	20	20	20	20	20

* ICN Biochemicals Inc., Cleveland, OH, USA.
† United States Biochemical Co., Cleveland, OH, USA.
‡ BDH Chemicals, Toronto, ON, Canada.

subsequently removed from samples by diethyl ether extraction for determination of organic PO_4^{3+} (Chen *et al.* 1956) and peptide (Markwell *et al.* 1978) contents. Contents of ileal wash were analysed for PEG (Malawer & Powell, 1967).

Bone biomechanical measurements. After the rats were killed, both femora were excised and cleansed of adhering soft tissue. Epiphyses were carefully removed without damaging tibia or femora. The left femur was consistently used in a biomechanical three-point bending analysis using an Instron Universal Testing Machine (Model 1122; Instron Corp., Canton, MA, USA; Yuan & Kitts, 1992*b*). Femora were bent until broken, by lowering a centrally placed point at a constant crosshead speed (1·0 mm/min).

The time–force deformation data from testing the femora in three-point bending were collected using the JCL 6000 Chromatography Data System (Jones Chromatography Ltd., Littleton, CO, USA) which was interfaced with the Instron, through a personal computer (IBM-AT compatible). Sample test time was 3 min, at a sampling rate of 5 signals/s. The Instron signal was calibrated with known weights of 1·0 and 2·0 kg, allowing data to be analysed by converting the millivolt signal output into kg force.

A number of whole-bone properties were determined from the three-point bending procedure. These were defined as bioyield, the force at which the first inflection in the initial slope of the time-force deformation curve occurs, expressed in Newtons (N); peak force, the maximum force applied during the bending procedure (N); bending failure energy, the amount of work energy (area under the time-force deformation curve) necessary to break the bone in bending, expressed in Joules; and lastly, maximum bending stress (σ), a force value that has been normalized to take into consideration bone size (N/mm^2; Yuan & Kitts, 1992*b*). This variable was calculated as follows:

$$\sigma = \frac{8 \times \text{maximum bending load } (L-1)\ D}{\pi(D^4 - d^4)},$$

where L is the distance between the supports (13 mm), D and d are the outer and inner diameters of the bone (mm).

Bone mineralization. The deposition of ^{45}Ca in the femur was determined from the right femur immediately after killing the rat. Femur mineral content was also determined from the right femur. Bone samples were dried (100°, 3 d), and then ashed (550°, 24 h). The bone ash was solubilized in 3 ml 4 M-HCl and portions taken for ^{45}Ca radioactivity and mineral content analyses (^{40}Ca, Mg and PO_4). Portions of solubilized bone ash were diluted with $LaCl_3$ (5 g/l) for Ca and Mg analyses by atomic absorption spectrophotometry. Solubilized bone wash was diluted with deionized water for determination of P by the colorimetric method of Chen *et al.* (1956).

Statistical analyses

All data are expressed as means and standard errors of the mean. One-way analysis of variance (for bone biomechanical variables presented in Figures 2 and 3 only) was used to test for differences between the experimental treatments (ANOVA; SPSS Inc., Chicago, IL, USA). Where differences did exist, the Student–Newman–Keuls multiple range test was used to identify the sources of the differences at a significance level of $P < 0{\cdot}05$. Significant differences ($P < 0{\cdot}05$) caused by protein source (S, casein or soya bean), protein quantity fed (R, 200 g/kg or low (heated or 60 g/kg)) and method of protein reduction (M, lowering proportion in diet or heating), inclusive of all experimental measurements, were analysed by two-way ANOVA (MANOVA; SPSS Inc.). Interactions (significance of F values at $P < 0{\cdot}05$) between protein source and dietary level fed (S × R) and protein source and method of reduction of protein (S × M) were also determined by two-way ANOVA.

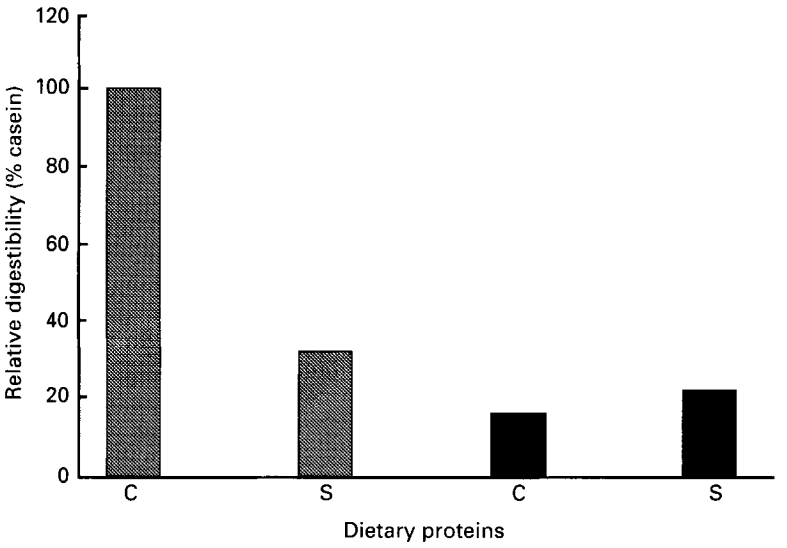

Fig. 1. Pepsin–pancreatin digestion estimates of heat damaged (■) casein (C), and soya-bean-protein isolate (S) and native (▨) casein and soya-bean-protein isolate calculated from the initial reaction rates (0–10 min). Respective slopes were: native casein $35{\cdot}5 \times 10^{-3}$; native soya-bean-protein isolate $11{\cdot}5 \times 10^{-3}$; heat damaged casein $5{\cdot}5 \times 10^{-3}$; heat damaged soya-bean-protein isolate $7{\cdot}5 \times 10^{-3}$; regression range $0{\cdot}983 \geqslant r \leqslant 0{\cdot}944$.

RESULTS

In vitro *digestibility study*

In vitro digestibility estimates for native and HD casein and soya-bean protein isolate respectively are shown in Fig. 1. Products of digestion, which are known to accumulate and inhibit the process of enzymic digestion (Robbins, 1978), were not removed. This disadvantage, however, is alleviated through the use of the initial slope method. A slower rate of proteolytic digestion was observed for native soya-bean protein compared with casein. Heat treatment of both protein sources resulted in a darker colour and marked decreases in their respective digestibilities.

In vivo *study*

Final body weight, dry matter intake and feed efficiency ratio (FER) of rats given HD or 60 g/kg protein diets were decreased ($P < 0{\cdot}05$) in comparison with control animals given 200 g protein/kg diet (Table 2). Dietary protein source had a significant ($P < 0{\cdot}05$) effect on animal growth characteristics. Animals fed on soya-bean protein diets had lower ($P < 0{\cdot}05$) final body weights and FER when compared with casein-fed counterparts.

Food intake expressed on an animal body weight basis, during the 1·5 h meal-feeding period before ileal loop surgery, was greater ($P < 0{\cdot}05$) in animals fed on HD and 60 g/kg protein diets (Table 3). Postprandial ileal loop peptide contents following the 1 h incubation period were increased ($P < 0{\cdot}05$) in animals fed on HD protein diets. Ileal PEG contents were decreased ($P < 0{\cdot}05$) in animals fed on HD protein diets.

The effects of feeding diets with different protein source and treatment to rats on the disappearance of ileal ^{45}Ca and deposition of tracer in femoral tissue are presented in Table 4. Dietary protein source and treatment significantly ($P < 0{\cdot}05$) affected ^{45}Ca disappearance from the ligated ileal loop. The percentage of ^{45}Ca lost from the ligated ileal loop was lower ($P < 0{\cdot}05$) in animals fed on soya-bean protein diets and HD casein. This observation with

Table 2. *Body weight, dry matter intake and feed efficiency ratio of spontaneously hypertensive rats fed on diets containing heat damaged protein, 60 g protein/kg, or 200 g protein/kg (control)**

(Mean values with their standard errors for thirty-six rats)

	Casein protein diets						Soya-bean-protein diets						
	Heat damaged		60 g protein/kg		200 g protein/kg (control)		Heat damaged		60 g protein/kg		200 g protein/kg (control)		Significant treatment effects†
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	
Initial body weight‡ (g)	77	3	82	2	77	2	78	3	88	3	79	2	—
Final body weight§ (g)	179	3	187	8	259	8	120	4	159	6	225	5	S, R, M, S × M
Dry matter intake‖ (g)	733	17	659	14	867	23	652	36	733	31	861	14	R, M
Feed efficiency ratio	0·140	0·004	0·160	0·004	0·211	0·004	0·065	0·005	0·097	0·004	0·170	0·004	S, R, M, S × R, S × M.

* For details of diets and procedures, see Table 1 and pp. 584–585.

† Significant ($P < 0·05$) treatment effects and interactions, where S is protein source (casein *v.* soya-bean protein), R is reduction (200 *v.* 60 g protein/kg and heat damaged), M is method of reduction (60 g protein/kg *v.* heat damaged).

‡ 5 weeks of age.

§ 14 weeks of age.

‖ Cumulative feed intake from 5 to 14 weeks of age.

Table 3. *Food intake and subsequent ileal loop peptide, organic phosphorus and polyethylene glycol (PEG) conents of spontaneously hypertensive rats fed on diets containing heat damaged protein, 60 g protein/kg, or 200 g protein/kg (control) at 14 weeks of age**

(Mean values with their standard errors for thirty-six rats)

	Casein protein diets						Soya-bean-protein diets						
	Heat damaged		60 g protein/kg		200 g protein/kg (control)		Heat damaged		60 g protein/kg		200 g protein/kg (control)		Significant treatment effects†
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	
Food intake‡ (g/kg body wt)	43·9	3·7	42·9	3·0	35·5	4·2	45·1	2·7	43·6	1·9	31·1	3·7	R
Total peptide§ (mg/loop)	25·2	0·3	16·4	0·7	16·0	0·4	20·5	0·3	14·4	0·2	14·6	0·5	M
Organic PO_4§ (μg/loop)	236	11	124	13	356	25	ND		9·5	2·1	24·6	6·1	S, M, R
PEG§ (mg/loop)	12·9	1·1	18·1	0·5	18·4	1·3	11·5	0·5	17·6	0·7	18·7	1·1	M

ND, none detected.

* For details of diets and procedures, see Table 1 and pp. 584–587.

† Significant ($P < 0·05$) treatment effects and interactions, where S is protein source (casein *v.* soya-bean protein), R is reduction (200 *v.* 60 g protein/kg and heat damaged), M is method of reduction (60 g protein/kg *v.* heat damaged).

‡ Food intake during 1·5 h meal-feeding period before surgery.

§ Ileal loop contents at killing.

Table 4. *Paracellular ^{45}Ca absorption from the ligated ileal loop of spontaneously hypertensive rats fed on diets containing heat damaged protein, 60 g protein/kg, or 200 g protein/kg (control) at 14 weeks of age**

(Mean values with their standard errors for thirty-six rats)

	Casein protein diets						Soya-bean-protein diets						
	Heat damaged		60 g protein/kg		200 g protein/kg (control)		Heat damaged		60 g protein/kg		200 g protein/kg (control)		Significant treatment effects†
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	
Intestine													
^{40}Ca (mg/loop)	2·26	0·41	2·46	0·31	2·25	0·22	2·02	0·39	2·39	0·33	2·16	0·20	—
^{45}Ca SA (dpm/mg ^{40}Ca)	5·08	0·65	3·39	0·44	4·30	0·70	5·39	0·49	3·64	0·26	4·60	0·30	—
^{45}Ca absorbed‡ (% dose)	20·4	2·6	42·1	3·8	43·2	2·0	22·6	4·8	25·7	2·7	29·2	3·4	S, R
Soluble ^{45}Ca§ (%)	87·6	3·1	49·2	4·4	57·8	3·9	86·3	3·4	31·7	2·0	35·9	2·1	S, R, S × R
Femur													
^{40}Ca in bone (mg/bone)	64·60	4·03	63·90	2·22	94·60	3·85	51·20	4·26	58·03	4·58	78·75	3·21	S, R, S × R
^{45}Ca SA (dpm/mg ^{40}Ca)	112·32	12·43	116·03	10·44	49·10	7·51	109·23	7·94	110·95	9·17	89·49	6·75	—
^{45}Ca deposited (% dose/bone)	0·160	0·010	0·151	0·018	0·104	0·017	0·111	0·007	0·126	0·012	0·148	0·008	—

SA, specific activity; dpm, disintegrations/min.

* For details of diets and procedures, see Table 1 and pp. 584–587.

† Significant ($P < 0·05$) treatment effects and interactions, where S is protein source (casein *v.* soya-bean protein), R is reduction (200 *v.* 60 g protein/kg and heat damaged), M is method of reduction (60 g protein/kg *v.* heat damaged).

‡ ^{45}Ca absorption (% dose) = [1 − ^{45}Ca (dpm) at 1 h/dose ^{45}Ca (dpm) administered] × 100.

§ Proportion of total ileal loop radioactivity remaining as soluble calcium after 1 h:

$$(\%) = \frac{\text{dpm } ^{45}Ca \text{ in supernatant from ileal loop}}{\text{total dpm in loop}} \times 100.$$

Table 5. *Mineral balance (24 h) of spontaneously hypertensive rats fed on diets containing heat damaged protein, 60 g protein/kg or 200 g protein/kg (control) at 10 weeks of age**

(Mean values with their standard errors for thirty-six rats)

	Casein protein diets						Soya-bean-protein diets						
	Heat damaged		60 g protein/kg		200 g protein/kg (control)		Heat damaged		60 g protein/kg		200 g protein/kg (control)		Significant treatment effects†
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	
24 h excretion													
Urine volume (ml/d)	8·0	0·8	5·6	0·9	13·1	0·6	7·5	0·6	6·2	0·6	15·8	0·2	—
Faeces dry wt (g/d)	0·78	0·10	0·81	0·06	1·50	0·22	0·79	0·10	1·33	0·07	1·25	0·06	—
Minerals													
Ca													
Intake (mg/d)	46·88	4·13	61·25	4·27	68·75	1·25	45·00	2·28	76·88	2·77	66·25	5·54	M
Urinary (mg/d)	1·93	0·11	0·45	0·04	1·11	0·14	0·67	0·08	0·54	0·06	0·94	0·04	S, R, M, S × R, S × M
Faecal (mg/d)	19·83	2·07	31·69	0·71	38·95	3·84	24·34	3·08	38·30	2·28	39·88	1·46	S, R, M
Balance‡ (mg/d)	23·1	3·2	33·4	3·5	33·5	2·5	19·9	1·6	36·7	2·0	28·6	3·1	S, M, S × R
Apparent absorption (%)	54·1	5·3	52·6	4·3	41·1	1·5	53·9	4·6	49·2	1·2	40·0	2·1	R
Mg													
Intake (mg/d)	4·69	0·41	6·12	0·43	6·88	0·12	4·50	0·23	7·69	0·28	6·62	0·55	M
Urinary (mg/d)	2·67	0·18	2·08	0·18	1·91	0·03	1·35	0·12	0·75	0·07	0·58	0·06	S, R, M
Faecal (mg/d)	0·48	0·05	0·97	0·06	1·49	0·10	0·66	0·05	1·76	0·16	2·29	0·03	S, R, M
Balance‡ (mg/d)	1·5	0·2	3·8	0·3	3·2	0·3	2·5	0·2	5·4	0·4	3·8	0·3	S, R, M
Apparent absorption§ (%)	89·4	0·8	83·7	2·2	71·2	7·1	87·4	1·7	77·5	2·9	67·8	2·5	S, R, M
PO_4													
Intake (mg/d)	55·60	3·78	58·12	2·06	79·48	1·44	56·42	2·35	72·88	2·45	84·38	5·48	R
Urinary (mg/d)	15·21	2·07	13·45	1·76	21·74	1·05	16·21	0·95	12·52	1·01	17·82	1·36	R
Faecal (mg/d)	23·31	2·19	16·26	1·45	23·61	2·18	17·09	1·22	26·76	1·79	22·18	1·31	—
Balance‡ (mg/d)	26·8	3·3	28·4	2·9	34·1	3·5	22·9	1·9	33·6	2·7	44·4	4·4	R, M
Apparent absorption§ (%)	72·5	3·5	70·2	3·6	68·4	3·0	69·8	1·4	62·5	3·5	72·5	2·2	—

* For details of diets and procedures, see Table 1 and pp. 584–587.

† Significant ($P < 0·05$) treatment effects and interactions, where S is protein source (casein *v.* soya-bean protein), R is reduction (200 *v.* 60 g protein/kg and heat damaged), M is method of reduction (60 g protein/kg *v.* heat damaged).

‡ Balance (mg/d) = intake − (urine + faecal). § Apparent absorption (%) = [(intake − faecal)/intake] × 100.

Table 6. *Femur physical characteristics and mineral composition of spontaneously hypertensive rats fed on diets containing heat damaged protein, 60 g protein/kg or 200 g protein/kg (control) at 14 weeks of age**

(Mean values with their standard errors for thirty-six rats)

	Casein protein diets						Soya-bean-protein diets						
	Heat damaged		60 g protein/kg		200 g protein/kg (control)		Heat damaged		60 g protein/kg		200 g protein/kg (control)		Significant treatment effects†
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	
Dry wt (g)	0·32	0·01	0·30	0·01	0·39	0·01	0·24	0·01	0·27	0·02	0·36	0·01	S, R, S × R, S × M
Ash wt (g)	0·18	0·01	0·16	0·01	0·24	0·01	0·12	0·01	0·15	0·01	0·21	0·01	S, R, S × R, S × M
Length (mm)	29·2	0·2	27·8	0·3	30·6	0·5	25·6	0·4	27·1	0·6	30·5	0·2	S, R, S × R, S × M
Ca (mg/bone)	64·6	4·03	63·9	2·22	94·6	3·85	51·2	4·26	58·0	4·58	78·8	3·21	S, R, S × R
Mg (mg/bone)	1·32	0·06	1·33	0·03	1·84	0·06	1·14	0·13	1·20	0·09	1·62	0·07	R
Ca:PO_4 ratio	1·77	0·14	2·10	0·05	2·00	0·01	1·82	0·04	2·00	0·03	1·97	0·05	—

* For details of diets and procedures, see Table 1 and pp. 584–587.

† Significant ($P < 0·05$) treatment effects and interactions, where S is protein source (casein *v.* soya-bean protein), R is reduction (200 *v.* 60 g protein/kg and heat damaged), M is method of reduction (60 g protein/kg *v.* heat damaged).

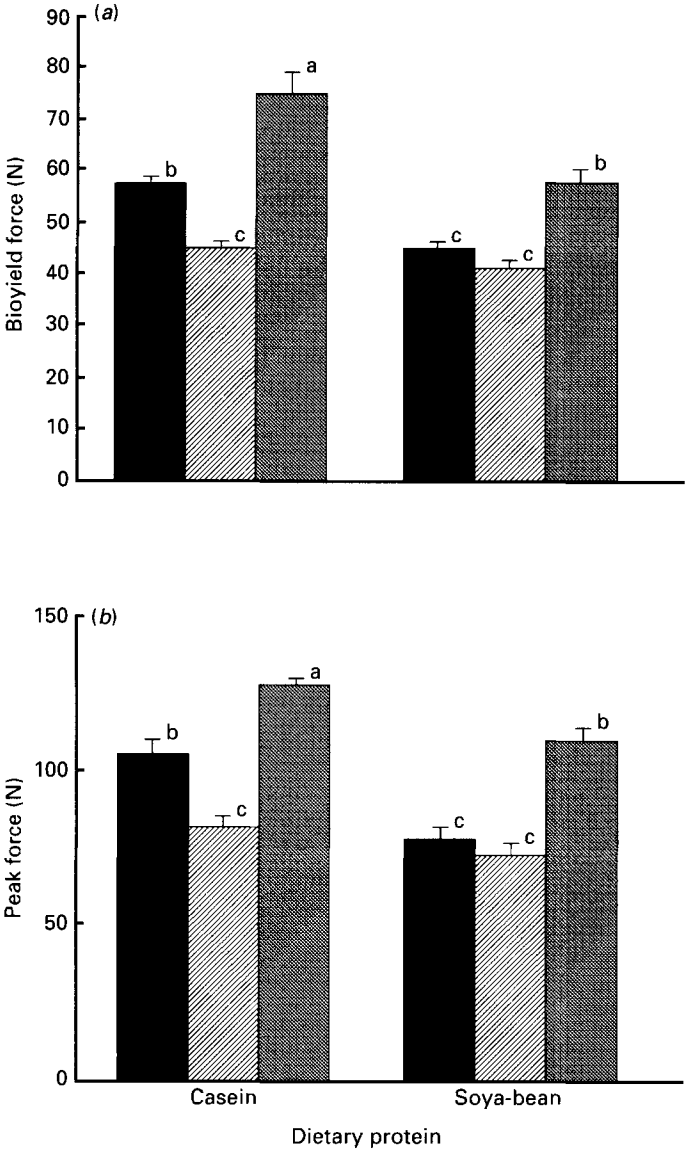

Fig. 2. Femur biomechanical force properties in spontaneously hypertensive rats (SHR) fed on heat damaged (■) casein and soya-bean-protein isolate, 60 g/kg (▨) and 200 g/kg (control; ▩) casein and soya-bean-protein isolate diets. (*a*) Femur bioyield force; (*b*) Femur peak force. [a,b,c] Means with different superscript letters were significantly different ($P < 0.05$).

HD casein-fed animals was associated with a relatively greater proportion of soluble ^{45}Ca in the ileal contents, and a higher organic P and peptide content compared with 60 g/kg casein-fed animals. Femoral ^{45}Ca specific activity was greater ($P < 0.05$) in animals fed on HD and 60 g/kg protein diets in comparison with those fed on the control diets.

The 24 h mineral balance study results are presented in Table 5. The volume of urine excreted by animals fed on HD and 60 g/kg protein diets during the 24 h collection was decreased ($P < 0.05$) compared with the control groups. The reduced feed intake of animals

Table 7. *Summary of dietary treatment effects on femur biomechanical properties**

Property measured†	Independent treatment effects‡	Treatment interactions§
Peak force	S, M, R	S × M, S × R
Bioyield	S, M, R	S × M, S × R
Maximum bending stress	S, M, R	—
Bending failure energy	S, R	S × R

* Analysed by two-way ANOVA ($P < 0.05$).

† For details of diets and properties measured see Table 1 and pp. 584–587.

‡ S is protein source (casein *v.* soya-bean protein), R is reduction of protein (200 *v.* 60 g protein/kg and heat damaged), M is method of reduction (60 g protein/kg *v.* heat damaged).

§ Interactions where S × R is protein source and protein reduction, S × M is protein source and method of reduction.

fed on HD proteins resulted in decreased ($P < 0.05$) Ca, Mg and P intakes. Urinary Ca excretion was significantly ($P < 0.05$) influenced by dietary protein source, dietary level and method of dietary reduction. Animals fed on HD and control casein diets excreted greater ($P < 0.05$) amounts of Ca in the urine than counterparts fed on soya-bean diets. Dietary protein source did not affect 24 h Ca balance or apparent absorption. Apparent absorption of Ca was enhanced ($P < 0.05$) in animals fed on HD and 60 g/kg protein diets. There were no interactive effects between source and level of protein intake on Ca apparent absorption.

An increase ($P < 0.05$) in urinary Mg excretion was observed in animals fed on both native and HD casein proteins (Table 5). Mg balance was reduced ($P < 0.05$) in animals fed on HD protein diets. Conversely, the apparent absorption of Mg was enhanced ($P < 0.05$) in animals fed on HD protein diets. There were no significant interactions between dietary treatments for Mg apparent absorption.

Diets were balanced for Ca content but not for P, and as a result, animals fed on the 60 g/kg casein diet had a lower PO_4 ($P < 0.05$) intake compared with control casein and 60 g/kg and control soya-bean protein-fed groups (Table 5). The apparent absorption of PO_4 was not influenced by dietary protein source or treatment.

The effects of feeding HD, 60 g/kg and control casein and soya-bean protein diets respectively on femur physical variables and mineral composition are reported in Table 6. Femur dry weight and ash weight were significantly ($P < 0.05$) affected by dietary protein source and level fed, but not influenced by the method of protein reduction. Protein source and level fed to SHR exhibited significant ($P < 0.05$) interactive effects for femoral weight and length. Femur Ca content was decreased ($P < 0.05$) in animals fed on the control soya-bean protein diet when compared with casein-fed counterparts. In addition, both HD and 60 g/kg protein-fed groups exhibited decreased ($P < 0.05$) femur calcification which resulted in a significant ($P < 0.05$) interaction between dietary protein source and level fed for bone calcification. Femoral Mg content was also reduced ($P < 0.05$) in HD and 60 g/kg protein groups. However, the method of protein reduction did not significantly influence femoral Ca or Mg contents. Significant interactions between source and reduction method of dietary protein for femoral ash weight and mineral content were not observed.

Femur bioyield and peak force values were lower ($P < 0.05$) in control soya-bean protein-fed animals compared with casein-fed counterparts (Fig. 2). Feeding HD and 60 g/kg protein diets resulted in reduced ($P < 0.05$) femur bioyield and peak force values. Interactions were obtained between protein source and level fed ($P < 0.05$), as well as protein source and reduction method ($P < 0.05$) for femur bioyield and peak force (Fig. 2

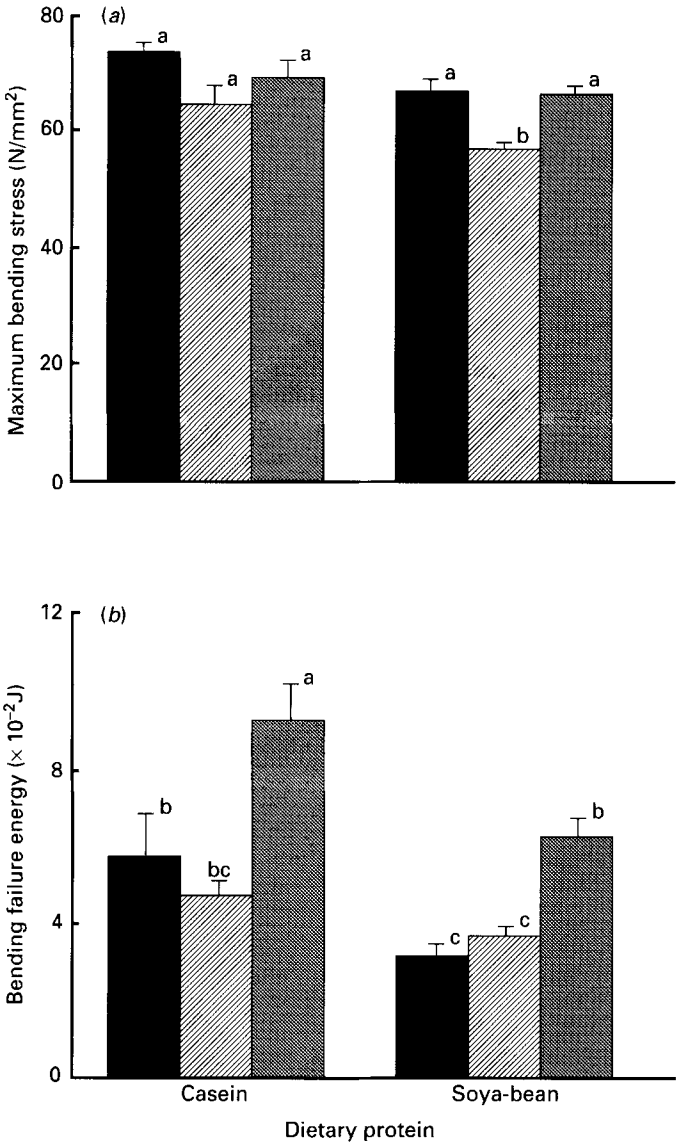

Fig. 3. Normalized femur biomechanical force and energy properties in spontaneously hypertensive rats (SHR) fed on heat damaged (■) casein and soya-bean-protein isolate, 60 g/kg (▨) and control 200 g/kg (▩) casein and soya-bean-protein isolate diets. (*a*) Femur maximum bending stress; (*b*) femur bending failure energy. [a,b,c] Means with different superscript letter were significantly different ($P < 0{\cdot}05$).

and Table 7). Conversely, protein source and the method of reduction did not exhibit an interactive effect on the normalized femur biomechanical force variable, maximum bending stress (Fig. 3 and Table 7). Animals fed on the control soya-bean protein diet exhibited a lower ($P < 0{\cdot}05$) bending failure energy compared with casein-fed counterparts. Femur bending failure energy was also decreased ($P < 0{\cdot}05$) in animals fed on HD and 60 g/kg protein diets. A significant ($P < 0{\cdot}05$) interactive effect between dietary protein source and level fed to animals was observed for femur bending failure energy.

DISCUSSION

Protein digestibility and yield of bioactive peptides

The reduced body weight gain and FER of SHR fed on soya-bean protein compared with casein concurs with the work of others who attribute this response to the reduced protein digestibility and lysine content of soya-bean protein (Lafevre & Schneeman, 1984). The similarity in lower body weight gained and FER of animals fed on HD and 60 g/kg protein diets indicates that the protein value of HD diets was comparable with that of a protein-deficient diet. The reduced nutritional value of HD proteins may be largely explained by the lower protein digestibility observed in the *in vitro* experiment. The severe heat sterilization treatment used in the present study would reduce protein digestibility by altering the tertiary structure of the proteins through the formation of isopeptides between lysine and arginine side chains and the carboxyl groups of aspartic and glutamic acids (Mauron, 1972). Moreover, the greater peptide concentration in ileal loops of animals fed on the HD proteins is a further reflection of a slower rate of digestion of both dietary and secretory proteins *in vivo*, which is not demonstrated in a simple *in vitro* system. For example, proteolytic activity in the intestinal contents is affected not only by pancreatic exocrine output but also by factors influencing intestinal secretion and intraluminal activation of proteolytic enzymes (Green & Lyman, 1972). The darker colour of the casein protein powder following thermal treatment suggests that a trace amount of lactose (a reducing sugar) was present, indicating the formation of non-enzymic Maillard browning reaction products (MRP). The loss of available lysine (Kanhai *et al.* 1987) combined with the reduced protein digestibility associated with MRP (Lee *et al.* 1977) are additional contributing factors to the reduced growth and nutrient malabsorption noted in this and other studies where animals were fed on HD proteins (Percival & Schneeman, 1979).

Lee *et al.* (1980) reported enhanced proteolytic activity and the production of only small amounts of macropeptides devoid of organic P in the intestinal contents of rats given a basal diet containing casein alone. This result was in contrast to the appreciable amounts of CPP present in the digesta of rats given a basal casein diet containing starch or fat. Thus, the production of bioactive CPP is influenced by factors regulating proteolytic activity and intestinal enzyme secretion. Therefore, in the present study it was hypothesized that reducing the digestibility of casein by heat denaturation would reduce the production of bioactive peptides, such as CPP, by decreasing turnover of pancreatic output and the protective effect of intestinal residue on enzymic autodigestion. Alternatively, animals fed on the 60 g/kg casein diet would quite feasibly produce a proportionally lesser amount of CPP than counterparts fed on a 200 g/kg casein diet. Although these experiments were not performed in pair-fed animals, which normally would have been preferred to eliminate the effects of variable feed intakes due to treatment differences, the present results indicate that intestinal CPP production was reduced in animals fed on 60 g/kg and HD casein diets. For example, based on the P content measured in ligated ileal loops of experimental animals and the amount of P present in CPP (60 mg P/g CPP; Lee *et al.* 1980), it can be calculated that the theoretical yield of CPP in animals fed on 200 g/kg, 60 g/kg and HD casein diets was 5·93 mg CPP, 2·07 mg CPP and 3·93 mg CPP, 1·5 h after ingestion of the respective diets. The yield of approximately 6 mg CPP in ileal contents of animals given the 200 g/kg casein diet agrees with previous estimates for a similar digestion period (Lee *et al.* 1980). Moreover, by feeding animals on 60 g/kg casein and HD casein diets we were able to reduce intestinal CPP production by 65 and 35% respectively, compared with the 200 g/kg casein diet.

CPP and calcium absorption

In vitro studies have demonstrated the formation of soluble Ca-chelates by CPP fragments (Reeves & Latour, 1958), as well as the inhibition of the formation of insoluble Ca–phosphate salts and thus, increased solubilization of Ca by CPP (Gerber & Jost, 1986). These characteristics confer bioactivity to CPP released by tryptic digestion of casein in enhancing paracellular absorption of Ca in rats by sequestering Ca ions within the intestinal milieu, thereby increasing the proportion of Ca available for absorption (Lee *et al.* 1980; Kitts *et al.* 1992). The greater effect of casein, observed herein, relative to soya-bean protein in enhancing ^{45}Ca disappearance from the ileum *in situ* is in agreement with previous studies from this laboratory (Nagasawa *et al.* 1991; Kitts *et al.* 1992). This observation is extended by results from the present study in that a reduced disappearance of ^{45}Ca was observed in both 60 and 200 g/kg soya-bean protein-fed animals compared with casein-fed counterparts. Moreover, animals fed on HD casein exhibited a similar *in situ* ^{45}Ca disappearance response to soya-bean-fed animals, which in turn was less than the 60 and 200 g/kg casein-fed animals. The similarity in the relative amounts of ileal ^{45}Ca absorbed from ligated loops of animals fed on 60 g/kg compared with 200 g/kg casein diets further suggests that the amount of CPP derived from the 200 g/kg casein diet could be in excess of that required to enhance paracellular Ca bioavailability. When taken together, these findings indicate that a minimal amount of CPP is required to enhance ileal ^{45}Ca absorption measured *in situ*. The form of Ca present in soya-bean protein isolates (i.e. phytic acid chelates) which has been shown to be relatively unavailable for absorption (Liebman & Landis, 1989) may also play a role in the lower ^{45}Ca absorption in the 60 g/kg and 200 g/kg soya-bean protein-fed animals compared with their casein-fed counterparts. Accordingly, other workers have recently demonstrated greater Ca and Mg bioavailability from bovine milk relative to soya-bean beverages (Brink *et al.* 1992).

The reduced ileal organic P content of the HD casein group, compared with 200 g/kg casein-fed animals is strong evidence for the thermally-induced β-elimination of PO_4 moieties from a portion of micellar phosphoseryl residues (Howat & Wright, 1934; Belec & Jenness, 1962). Dephosphorylation of caseins reduces Ca binding to CPP (Berrocal *et al.* 1989), thereby decreasing the *in situ* Ca solubilization activity (Sato *et al.* 1983*b*). The effects of thermal treatment on casein and the resultant decreased production of CPP paralleled the lower *in situ* paracellular Ca absorption in HD casein-fed animals. It is of interest that, despite a greater proportion of soluble ^{45}Ca recovered from ileal contents of rats fed on both HD protein diets, the disappearance of ^{45}Ca from the intestinal loop of these animals was reduced. This observation may result from the fact that heating whole casein can increase overall Ca binding potential by inducing conformational changes that result in the unmasking of additional Ca binding sites on the protein, such as carboxyl groups (Kitts *et al.* 1991; Pappas & Rothwell, 1991), while having no effect on Ca bioavailability. Naito *et al.* (1972) have previously demonstrated that various macropeptide species released from digestion of dietary proteins may not necessarily enhance Ca bioavailability through solubilization of this mineral. Caution is therefore required in equating increased intestinal Ca solubility *a priori* with enhanced bioavailability (Yuan & Kitts, 1991).

Mineral balance studies

The 24 h apparent absorption measurements for Ca, Mg and P are in agreement with those reported by others (Sato *et al.* 1986; Jones *et al.* 1988). During Ca deficiency, extracellular Ca homeostasis is regulated by urinary Ca excretion (Agus *et al.* 1981). However, this effect was not apparent in the present study, as urinary Ca losses were actually increased in animals fed on HD protein diets. Studies have shown that P has a direct positive effect on renal tubular Ca reabsorption (Zemel, 1988); therefore, P deficiency would result in

reduced tubular reabsorption of Ca and consequently increased urinary Ca excretion. A P deficiency has in fact been reported to result in increased urinary Ca excretion and negative Ca balance (Rader *et al.* 1979). Thus, it is noteworthy that animals fed on the HD casein diet exhibited lower 24 h Ca and P balances. Ca homeostatic mechanisms in HD protein-fed animals compensated for increased urinary Ca excretion with decreased faecal Ca loss and thereby increased Ca apparent absorption. Moreover, the similarity between the Mg and Ca balance data, reflecting the higher Mg apparent absorption and increased urinary excretion, also suggests that a homeostatic mechanism was in effect for Mg in HD protein-fed animals.

The disparate results obtained between *in situ* Ca absorption and 24 h Ca apparent absorption are attributed to differences in the physiological mechanisms regulating Ca absorption for these endpoint measurements used to quantitate Ca absorption. A ligated ileal segment allows estimation of the disappearance of ^{45}Ca from the distal small intestine via paracellular absorption across the intestinal mucosa. This pathway occurs along the length of the entire intestine and is susceptible to the influence of dietary factors (Wasserman & Taylor, 1976). On the other hand, estimates of Ca absorption from balance studies involve not only the absorptive capacity of the entire intestine (including the transcellular route, dominant in the duodenum and jejunum, as well as paracellular Ca movement) but also endogenous homeostatic mechanisms. Ca absorption via the transcellular pathway is subject to both nutritional and physiological regulation (Rader *et al.* 1979; Agus *et al.* 1981). It is apparent that the comprehensiveness of the balance study may mask the small localized changes in Ca bioavailability observed *in situ* which were attributable to the activity of CPP. A similar conclusion concerning the accuracy of the balance method in detecting small changes in intestinal Ca absorption attributed to the effect of lactose has recently been reported (Shortt & Flynn, 1991).

Bone physicochemical and biomechanical variables

The higher femur ^{45}Ca specific activities of groups fed on soya-bean protein diets as well as the HD and 60 g/kg casein diets consistently reflected the lower ^{40}Ca content of the femora from these animals. Other workers have used similar acute radiolabelled Ca uptake measurements to estimate Ca bioavailability (Sato *et al.* 1986) or more prolonged femur ^{45}Ca deposition protocols (Buchowski *et al.* 1989) to estimate Ca bioavailability from dairy foods. Considering the relatively short time-period used previously (Sato *et al.* 1986) and herein, it is possible that this measurement does not accurately reflect Ca utilization and deposition *per se*, but rather the physicochemical exchange of ^{45}Ca for ^{40}Ca on the surface of bone. Our results would suggest that the higher specific activity of ^{45}Ca in animals given the soya-bean protein diets, as well as those given the modified casein diets, represents an indication of increased bone turnover or a higher proportion of newly mineralized bone in these animals. This finding corroborates the reported higher bone turnover in rats given soya-bean beverage compared with those given cow's milk (Brink *et al.* 1992).

Early studies by Mellander *et al.* (1950, 1956) first reported that CPP increased bone calcification of rachitic subjects. Previous reports have indicated that CPP enhanced intestinal Ca absorption and utilization for skeletal deposition in chicks and rats (Mykkanen & Wasserman, 1980; Sato *et al.* 1986). The significance of a CPP effect on bone Ca content and strength variables was evaluated in the present study through treatment interactions between protein source and the two methods used to reduce CPP production. For example, both protein source (casein v. soya-bean protein) and reduced protein intake (200 g protein/kg v. low (heated and 60 g/kg)) resulted in independent effects on bone mineralization and biomechanical parameters. It is of interest, however, that the methods used to reduce protein intake (i.e. lowering the percentage of protein in the diet and heating) did not have an independent effect on bone mineralization, albeit the measurements

of biomechanical strength (namely, peak force and bioyield) were significantly affected. Even though a significant interaction for protein source and reduction in protein fed was found for bone calcification, the fact that a similar significant interaction between protein source and method of protein reduction was not obtained for this variable suggests that the role of CPP in enhancing Ca utilization could only be significant above a threshold level of CPP in the intestinal milieu. The present findings extend those of a previous report from this laboratory, wherein supplementation of a soya-bean protein diet with 3% CPP increased ileal Ca bioavailability but did not significantly enhance femur calcification in Ca-replete SHR (Yuan & Kitts, 1991). These findings confirm the limited role for CPP in enhancing Ca utilization, which is dependent on the amount and quality of casein fed. These results also suggest that nutrient malabsorption resulting from feeding HD proteins affected Ca homeostasis to an extent that bone Ca was not spared. For example, the reduction in bone Ca and Mg content in HD casein- and HD soya-bean protein-fed animals coincided with a lower P balance, which is consistent with the acute inhibition of bone mineralization observed in P-deficient animals (Bruin *et al.* 1975). Decreased bone length, weight and cortical thickness noted in the present study with animals fed on the HD protein diets has previously been reported to be symptomatic of protein malnutrition (Garn *et al.* 1964). A similar conclusion has also been reported from this laboratory in lactose-intolerant animals (Yuan *et al.* 1991).

Both protein source (casein v. soya-bean) and reduced protein intake (200 g/kg v. low (60 g/kg and HD)) yielded independent effects for all bone biomechanical variables tested. Bone consists of both the mineral content necessary for hardness and rigidity, as well as an organic collagen matrix which provides toughness and elasticity. The biomechanical force properties, peak force and bioyield, are characteristic of the mineralization of bone, and thus of bone hardness. Alternatively, the work energy required to break the bone, bending failure energy, is representative of the mineral:organic components ratio of bone (Yuan & Kitts, 1991). Animals fed on HD protein diets exhibited femur biomechanical properties that were characteristic of smaller bone size, as evidenced by lower bone mineralization and reduced content of matrix components. Other studies have related bone strength to bone mineral content and ash weight (Crenshaw, 1986). In the present study the two methods used to lower protein intake (60 g/kg and HD proteins) had independent effects on peak force, bioyield and maximum bending stress, but not on the reduced bending failure energy required to break the bones in treatment animals, relative to 200 g/kg controls. These results demonstrate that while the effect of reducing CPP (a feature common to both treatments) was a factor in bone hardness measurements, it did not influence the inorganic:organic material ratio in the bones. On the other hand, the significant interactive effects obtained between protein source and method of reducing protein intake (60 g/kg and HD proteins) for both peak force and bioyield, but not maximum bending stress or bending failure energy, can be interpreted as an indication of changes occurring in bone tissues that were secondary to nutrient malabsorption and protein deficiency, rather than loss of CPP activity. The signs of reduced bone mass observed in both 60 g/kg and HD protein-fed animals have previously been associated with protein deficiency-derived malnutrition (Garn *et al.* 1964; Adams & Berridge, 1969). These observations are not consistent with other work with protein deficiency in rats which resulted in slower growth but normal bone mineralization (Orwoll *et al.* 1992).

In summary, it seems likely that the presence of CPP in the distal small intestine has a positive effect in enhancing Ca bioavailability in this region. The similar paracellular Ca absorption noted between animals fed on low (60 g/kg) and control casein diets was greater than in soya-bean protein-fed counterparts, and suggests that a more than adequate amount of CPP is generated to enhance Ca absorption from the distal small intestine.

Alternatively, a factor associated with soya-bean protein isolate effectively reduced Ca bioavailability. Despite the reduced protein digestibility and lower ileal Ca absorption in animals fed on HD casein, metabolic homeostatic mechanisms attempted to maintain Ca balance not only by increased apparent absorption of dietary Ca, but also at the cost of femoral Ca content and specific bone strength properties. The battery of methods used to assess the role of CPP in facilitating Ca bioavailability differed in their relative sensitivities in determining whether CPP enhanced Ca intestinal bioavailability and subsequent utilization.

This study was supported by research grants from Dairy Bureau of Canada and NSERC.

REFERENCES

Adams, P. & Berridge, F. R. (1969). Effects of kwashiorkor on cortical and trabecular bone. *Archives of Disease in Childhood* **44**, 705–709.

Agus, Z. S., Wasserstein, A. & Goldfarb, S. (1981). PTH, calcitonin, cyclic nucleotides, and the kidney. *Annual Review of Physiology* **41**, 583–595.

Belec, J. & Jenness, R. (1962). Dephosphorylation of casein by heat treatment. II. In skimmilks. *Journal of Dairy Science* **45**, 20–26.

Berrocal, R., Chanton, S., Juillerat, M. A., Pavillard, B., Scherz, J. C. & Jost, R. (1989). Tryptic phosphopeptides from whole casein. II. Physicochemical properties related to the solubilization of calcium. *Journal of Dairy Research* **56**, 335–341.

Brink, E. J., Dekker, P. R., Van Beresteijn, E. C. H. & Beynen, A. C. (1992). Bioavailability of magnesium and calcium from cow's milk and soya-bean beverage in rats. *British Journal of Nutrition* **68**, 271–282.

Brommage, R., Juillerat, M. A. & Jost, R. (1991). Influence of casein phosphopeptides and lactulose on intestinal calcium absorption in adult female rats. *Le Lait* **71**, 173–180.

Bruin, W. J., Baylink, D. J. & Wergedal, J. (1975). Acute inhibition of mineralization and stimulation of bone resorption mediated by hypophosphatemia. *Endocrinology* **96**, 394–399.

Buchowski, M. S., Sowizral, K. C., Lengemann, F. W., Van Campen, D. & Miller, D. D. (1989). A comparison of intrinsic and extrinsic tracer methods for estimating calcium bioavailability in rats from dairy foods. *Journal of Nutrition* **119**, 228–234.

Canadian Council on Animal Care (1984). *Guide to the Care and Use of Experimental Animals*, vol. 2. Ottawa, ON: Canadian Council on Animal Care.

Chen, P. S., Toribara, T. Y. & Warner, H. (1956). Microdetermination of phosphorus. *Analytical Chemistry* **28**, 1756–1758.

Crenshaw, T. D. (1986). Reliability of dietary Ca and P levels and bone mineral content as predictors of bone mechanical properties at various periods in growing swine. *Journal of Nutrition* **116**, 2155–2170.

Dawson-Hughes, B., Jacques, P. & Shipp, C. (1987). Dietary calcium intake and bone loss from the spine in healthy post menopausal women. *American Journal of Clinical Nutrition* **46**, 685–687.

Garn, S. M., Rohmann, C. G., Behar, M., Viteri, F. & Guzman, M. A. (1964). Compact bone deficiency in protein-calorie malnutrition. *Science* **145**, 1444–1445.

Gerber, H. W. & Jost, R. (1986). Casein phosphopeptides: their effect in calcification of in vitro cultured embryonic rat bone. *Calcified Tissue International* **38**, 350–357.

Green, G. M. & Lyman, R. L. (1972). Feedback regulation of pancreatic enzyme secretion as a mechanism for trypsin inhibitor induced hypersecretion in rats. *Proceedings of the Society for Experimental Biology and Medicine* **140**, 6–12.

Heaney, R. P., Gallagher, J. C., Johnson, C. C., Neer, R., Parfitt, A. M. & Whedon, G. D. (1982). Calcium nutrition and bone health in the elderly. *American Journal of Clinical Nutrition* **36**, 986–1013.

Howat, G. R. & Wright, N. C. (1934). The heat-coagulation of caseinogen. I. The role of phosphor cleavage. *Biochemical Journal* **28**, 1336–1345.

Itaya, K. & Ui, M. (1966). A new micromethod for the colorimetric determination of inorganic phosphate. *Clinica Chimica Acta* **14**, 361–366.

Izawa, Y., Sagara, K., Kadota, T. & Makita, T. (1985). Bone disorders in spontaneously hypertensive rat. *Calcified Tissue International* **37**, 605–607.

Jacques, H., Deshaies, Y. & Savoie, L. (1986). Relationship between dietary proteins, their in vitro digestion products and serum cholesterol in rats. *Atherosclerosis* **61**, 89–98.

Jones, M. R., Martins, J. E. & Clemens, R. A. (1988). Mineral balance and blood pressure in the young spontaneously hypertensive rat. *Journal of Nutrition* **118**, 114–120.

Kanhai, J., Kitts, D. D. & Powrie, W. D. (1987). Temporal changes in serum cholesterol levels of rats fed casein and skim milk powdered diets. *Journal of Food Science* **52**, 1410–1413.

Kitts, D. D., Leung, R. & Nakai, S. (1991). Extrinsic labelling of caseinophosphopeptides with ^{45}calcium and recovery following thermal treatment. *Canadian Institute of Food Science and Technology Journal* **24**, 278–282.
Kitts, D. D., Yuan, Y. V., Nagasawa, T. & Moriyama, Y. (1992). Effect of casein, casein phosphopeptides and calcium intake on ileal ^{45}Ca disappearance and temporal systolic blood pressure in spontaneously hypertensive rats. *British Journal of Nutrition* **68**, 765–781.
Kwan, K. K. H., Nakai, S. & Skura, B. J. (1983). Comparison of four methods for determining protease activity in milk. *Journal of Food Science* **48**, 1418–1421.
Lafevre, M. & Schneeman, B. O. (1984). High density lipoprotein composition in rats fed casein and soy protein isolate. *Journal of Nutrition* **114**, 768–777.
Lee, C. M., Chichester, C. O. & Lee, T.-C. (1977). Effect of Maillard reaction products on disaccharidase activities in the rat. *Journal of Agricultural and Food Chemistry* **25**, 775–778.
Lee, Y. S., Noguchi, T. & Naito, H. (1980). Phosphopeptides and soluble calcium in the small intestine of rats given a casein diet. *British Journal of Nutrition* **43**, 457–467.
Liebman, M. & Landis, W. (1989). Calcium and zinc balances of premenopausal women consuming tofu-compared to cheese-containing diets. *Nutrition Research* **9**, 5–14.
Maga, J. A., Lorenz, K. & Onayemi, O. (1973). Digestive acceptability of proteins as measured by the initial rate of in vitro proteolysis. *Journal of Food Science* **38**, 173–174.
Malawer, S. J. & Powell, D. W. (1967). An improved turbidometric analysis of polyethylene glycol utilizing an emulsifier. *Gastroenterology* **53**, 250–256.
Markwell, M. A., Hans, S. M., Bieber, L. L. & Tolbert, N. E. (1978). A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. *Analytical Biochemistry* **87**, 206–210.
Mauer, J. (1977). Extraction method for the simultaneous determination of Na, K, Ca, Mg, Fe, Cu, Zn, and Mn in organic material using atomic absorption spectrophotometry. *Zeitschrift fur Lebensmittel Untersuchung und Forschung* **165**, 1–4.
Mauron, J. (1972). Influence of industrial and household handling on food protein quality. In *Protein and Amino Acid Functions*, pp. 417–474 [E. J. Bigwood, editor]. Oxford: Pergamon Press.
Meisel, H. & Frister, H. (1989). Chemical characterization of bioactive peptides from in vivo digests of casein. *Journal of Dairy Research* **56**, 343–349.
Mellander, O. (1950). The physiological importance of the casein phosphopeptide calcium salts. II. Peroral calcium dosage of infants. *Acta Societatis Medicorum Upsaliensis* **55**, 247–255.
Mellander, O. & Olsson, N. (1956). The influence of peptide bound calcium and phosphorus in bone calcification in rickets. *Biologica Medicus Del Hospital De Mexico* **13**, 243–246.
Mykkanen, H. M. & Wasserman, R. H. (1980). Enhanced absorption of calcium by casein phosphopeptides in rachitic and normal chicks. *Journal of Nutrition* **110**, 2141–2148.
Nagasawa, T., Yuan, Y. V. & Kitts, D. D. (1991). Casein phosphopeptides enhance paracellular calcium absorption but do not alter temporal blood pressure in normotensive rats. *Nutrition Research* **11**, 819–830.
Naito, H., Kawakami, A. & Imamura, T. (1972). In vivo formation of phosphopeptide with calcium-binding property in the small intestinal tract of the rat fed on casein. *Agricultural and Biological Chemistry* **36**, 409–415.
Naito, H. & Suzuki, H. (1974). Further evidence for the formation in vivo of phosphopeptides in the intestinal lumen from β-casein. *Agricultural and Biological Chemistry* **38**, 1543–1545.
Orwoll, E., Ware, M., Stibrska, L., Bilke, D., Sanchez, T., Andon, M. & Li, H. (1992). Effects of dietary protein deficiency on mineral metabolism and bone mineral density. *American Journal of Clinical Nutrition* **56**, 314–319.
Pappas, C. P. & Rothwell, J. (1991). The effects of heating, alone or in the presence of calcium or lactose, on calcium binding to milk proteins. *Food Chemistry* **42**, 183–201.
Percival, S. S. & Schneeman, B. O. (1979). Long term pancreatic response to feeding heat damaged casein in rats. *Journal of Nutrition* **109**, 1609–1614.
Rader, J. I., Baylink, D. J., Hughes, M. R., Safilian, E. F. & Haussler, M. R. (1979). Calcium and phosphorus deficiency in rats: effects on PTH and 1,25-dihydroxyvitamin D_3. *American Journal of Physiology* **236**, E118–E122.
Reeves, R. E. & Latour, N. G. (1958). Calcium phosphate sequestering phosphopeptide from casein. *Science* **128**, 472.
Robbins, R. C. (1978). Effect of ratio of enzymes to substrate on amino acid patterns released from protein in vitro. *International Journal for Vitamin and Nutrition Research* **48**, 44–53.
Sato, R., Noguchi, T. & Naito, H (1983*a*). Effect of lactose on calcium absorption from the rat small intestine and a non-flushed ligated loop. *Journal of Nutritional Science and Vitaminology* **29**, 365–373.
Sato, R., Noguchi, T. & Naito, H. (1983*b*). The necessity for the phosphate portion of casein molecules to enhance calcium absorption from the small intestine. *Agricultural and Biological Chemistry* **47**, 2415–2417.
Sato, R., Noguchi, T. & Naito, H. (1986). Casein phosphopeptide (CPP) enhances calcium absorption from the ligated segment of rat small intestine. *Journal of Nutritional Science and Vitaminology* **32**, 67–76.
Shah, B. G., Belonje, B. & Paquet, A. (1990). The lack of effect of synthetic phosphoseryl peptide on calcium absorption by the rat. *Nutrition Research* **10**, 1331–1336.
Shortt, C. & Flynn, A. (1991). Effect of dietary lactose on salt-mediated changes in mineral metabolism and bone composition in the rat. *British Journal of Nutrition* **66**, 73–81.
Wasserman, R. H. & Taylor, A. N. (1976). Gastrointestinal absorption of calcium and phosphorus. In *Handbook*

of Physiology, vol. 7, pp. 137–155 [R. O. Greep and E. B. Astwood, editors]. Washington, DC: American Physiological Society.

Yuan, Y. V. & Kitts, D. D. (1991). Confirmation of calcium absorption and femoral utilization in spontaneously hypertensive rats fed casein phosphopeptide supplemented diets. *Nutrition Research* **11**, 1257–1272.

Yuan, Y. V. & Kitts, D. D. (1992*a*). Effect of dietary calcium intake and protein source on calcium utilization and bone biomechanics in the spontaneously hypertensive rat. *Journal of Nutritional Biochemistry* **3**, 452–460.

Yuan, Y. V. & Kitts, D. D. (1992*b*). Estimation of dietary calcium utilization in rats using a biomechanical functional test. *Food Chemistry* **44**, 1–7.

Yuan, Y. V., Kitts, D. D. & Nagasawa, T. (1991). The effect of lactose and fermentation products on paracellular calcium absorption and femur biomechanics in rats. *Canadian Institute of Food Science and Technology Journal* **24**, 74–80.

Yvon, M. & Pelissier, J. P. (1987). Characterization and kinetics of evacuation of peptides resulting from casein hydrolysis in the stomach of the calf. *Journal of Agricultural and Food Chemistry* **35**, 148–156.

Zemel, M. B. (1988). Calcium utilization: effect of varying level and source of dietary protein. *American Journal of Clinical Nutrition* **48**, 880–883.

Printed in Great Britain