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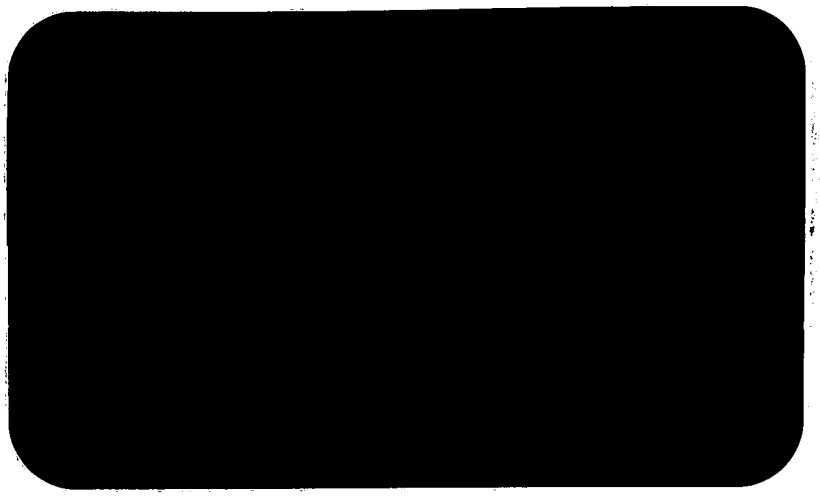

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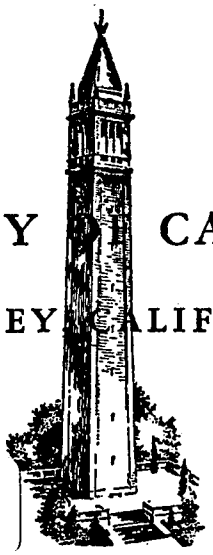

THE EFFECT OF VARIABLE CALCIUM AND VERY LOW CALCIUM DIETS ON HUMAN CALCIUM METABOLISM

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The Effect of Variable Protein and Very Low Calcium Diets on Human Calcium Metabolism

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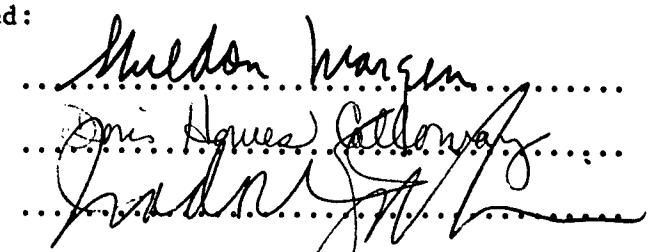

Committee in Charge

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INTRODUCTION

Calcium is the fifth most abundant element in the human body. Ninty-nine percent of calcium is found in bone and the rest in muscle, tissue, blood and extracellular fluid. Insoluble calcium as apatite is the most important structural element in the bone; however soluble calcium in the extraosseous tissues plays an essential role im many diverse physiological processes such as membrane integrity, enzymatic reactions, muscle contraction and nerve transmission. Highly integrated and efficient homeostatic mechanisms exist to maintain and regulate these dual roles -- mechanical and physiological.

The body is able to adapt to a wide variety of external changes and still maintains its integrity. Difference in nutritional intake is one of these major variables. Calcium metabolism is not only affected by the amount and form of calcium in the diet but also by many other dietary constituents.

Protein is required in the diet in order to provide the essential amino acids and nitrogen. The daily protein ingested varies both in quantity and quality for any individual especially between individuals. However, the effect of this most important dietary component on calcium metabolism has not been well studied.

More than half a century ago, Sherman (1) observed that addition of meat to a diet caused an increase in urinary calcium excretion. Milk, soybean curd, egg white, gelatin, peptone, gluten and other protein sources were also shown to influence urinary or fecal calcium by many other investigators (Table 3, page 32). These findings demonstrated that protein intake could affect calcium metabolism. Until recently, there was no well controlled human experiment to evaluate the relationship between protein intake and calcium metabolism. During a series of carefully controlled experiments using formula diets to study the metabolic responses to varying protein intake, Margen and Calloway[1,2] observed a direct relationship between protein intake and urinary calcium excretion. The increase in urinary excretion has been attributed, at least in part, to the enhancement of intestinal calcium absorption[3] (2).

Dietary surveys have demonstrated a wide variation in calcium intake between individuals, geographical areas and societies (3). A low calcium intake is very common in many Asian and African countries. The protein intake in these areas is generally low. In this country, about 85% of calcium intake comes from milk and dairy product (4). Therefore, the individual who does not like or tolerate milk and dairy products may have a high protein but a low calcium intake. A well controlled human

experiment to study the effect of diet containing very low calcium with either high or low protein has not been previously reported.

This experiment attempted to study the effect of very low calcium diet with variable levels of protein in order to simulate as well as exaggerate the two different conditions described above. ~~Because of the very low calcium intake,~~ this experiment was also designed to study the role of intestinal calcium absorption on urinary calcium excretion, rate of production of endogenously secreted calcium in the gastrointestinal tract, and the dynamics of calcium metabolism in order to elucidate the mechanisms of the calciuretic effect of high protein intake.

REVIEW OF LITERATURE

Calcium Metabolism

Although most of the calcium in the body is insoluble, calcium is still maintained in a dynamic state by continuous absorption, exchange, deposition and resorption in bone, excretion and other processes. The dynamics of calcium metabolism are complex. An understanding of calcium metabolism depends on the ability to measure these processes in the body. Many investigators have attempted to simplify this complex system by using certain schemes and mathematical formulas, but there has been no general agreement as to their validity as each model is probably incomplete.

Absorption: Dietary surveys based on selected areas and populations show that most people consume 200-1500 mg calcium per day (3). The major source of dietary calcium ranges from 88% from milk and dairy products in New Zealand to 72% from plant sources in the Philipines (4).

The ingested calcium is mixed in the gut with digestive juice calcium. Only a fraction of both ingested and endogenous calcium is absorbed. Some calcium is absorbed by diffusion and there is also an active transport mechanism for calcium absorption, but the relative significance of this active transport process is unclear (5, 6, 7 for rev.). Vitamin D is essential for this active

transport. The absorbed vitamin D is converted to 25-hydroxycholecaliferol in the liver. This metabolite is then transported back to the intestinal epithelium where it or its metabolites (8) exert a specific action on DNA. It initiates a transcription process that leads to the synthesis of a specific protein or proteins which are required for calcium active transport (7, 9, 10 for rev.). This protein or proteins may be the specific calcium binding protein of Wasserman et al. (7, 11, 12) and/or the calcium-dependent-ATPase of DeLuca (10).

The efficiency of calcium absorption differs in different segments of the intestine. Although the duodenum is the most efficient segment of the intestine in terms of unit length in almost all mammals except the hamster (13), the ileum is the most effective site for calcium absorption because of the longer residence time in this segment (14-16).

Aubert et al. (17) gave two schemes for calculating the true fractional absorption rate. The concept of endogenous fecal calcium is dependent on the choice of scheme. In the first scheme the endogenous fecal calcium is considered the calcium secreted into the gut after calcium absorption has taken place. The endogenous fecal calcium in this scheme is a parameter independent of calcium absorption and is a relatively constant value. However, the second scheme consideres the endogenous fecal

calcium as unreabsorbed digestive juice calcium. The digestive juice calcium is considered to be mixed homogenously with ingested calcium and both are absorbed at the same rate. Many factors can affect calcium absorption therefore the endogenous fecal calcium in the second scheme is a function of many factors.

Marshall (18) proposed another scheme and introduced an additional parameter, β, which represents the fraction of digestive juice calcium that is reabsorbed. He considered the β value to be between 0 and the fractional absorption rate of dietary calcium, α. However, studies on the absorption of digestive juice calcium showed somewhat different results. Heaney and Skillman (19) showed a non-absorbable fraction of endogenous secreted calcium. Schedl et al. (20) also found the digestive juice calcium to be less available for absorption because it is readily precipitated with secreted phosphate. Bristol and Regan (21), however, demonstrated preferential absorption of endogenous calcium from the bile. It appears then that the value of β may be larger or smaller than α.

Resolution of the schemes has been accomplished in several ways. Brine and Johnston (22) plotted dietary calcium against fecal calcium and extrapolated the curve to zero calcium intake. They considered the amount of fecal calcium at zero intake of calcium to be endogenous fecal calcium. Blau et al. (23, 24) were the first to

use radiocalcium in human subjects to determine these parameters. Radiocalcium can be administered orally and/or intravenously (17, 25). A double-isotope method has also been applied for this purpose (26-28). Fractional absorption rate and endogenous fecal calcium calculated by many investigators are shown in Table 1. Because of the different schemes, assumptions and methods, there is substantial difference between these estimations.

Another method for evaluating the efficiency of intestinal calcium absorption is to measure plasma activity after a large oral dose of radiocalcium (33-35). The peak of radioactivity in plasma is reached in one to two hours and ranges from 0.3% to 3.9% of the dose per liter of plasma. There is a good correlation between the two hour plasma activity and the net calcium absorption (33, 34).

The efficiency of calcium absorption is influenced by many factors. The mechanisms by which these factors affect the efficiency of calcium absorption are far from clear but are certainly diverse. They may induce, compete or inhibit the transport mechanism of calcium in the intestine, change the physicochemical properties of calcium salts, interfere with the metabolism of intestinal epithelial cells, and modify the general calcium metabolism.

Vitamin D is the most important factor for the active

Table 1

Fractional Calcium Absorption Rate

and Endogenous Fecal Calcium

Calcium Intake (V_i)	Fractional Absorption Rate (α)	Endogenous Fecal Calcium (V_{ef})	Method[a]	Reference
mg/day	%	mg/day		
1900	37-49[b]	186-425[b]	I.V.+P.O., N=5	(29)
722	27	33	I.V., N=2	(30)
616	35	94	"	
107 & 135	44 & 67	91 & 118	P.O., N=2	(24)
529 & 638	40 & 69	91 & 87	"	
1446&1740	54 & 61	73 & 93	"	
0- 199	38	75	From Literature[c]	(22)
200- 399	42	"		
400- 599	43	"		
600- 799	35	"		
800- 999	34	"		
1000-1199	28	"		
209	30.5	--	I.V.+P.O., N=21	(31)
2180	63.6	--	"	
200	67.9	--	I.V., N=4	(32)
800	40.1	--	I.V., N=6	
2000	23.3	--	I.V., N=4	
10mg/test	79.5±8.4[d]	--	I.V. (^{47}Ca)+ P.O. (^{45}Ca) N=28	(26)
200mg/test	25.6±7.9	--		
500mg/test	21.8±4.3	--		
--	--	130±47	I.V., N=36	(19)

[a] Radiocalcium administration, intravenously (I.V.) or orally (P.O.). N= number of subjects, all subjects were adults except those otherwise specified.

[b] Aldolescent boys, age 11-16 years old.

[c] See page 6 for the method of calculation.

[d] Mean and standard deviation.

transport of calcium. An understanding of its role at the molecular level has been well advanced in recent years (see page 4-5). Both sodium and potassium can inhibit intestinal calcium transport probably because of their competition for binding sites with calcium (7, 36, 37). Studies on the effect of magnesium on intestinal calcium transport are conflicting. Intestinal calcium absorption may increase, decrease or be unchanged by magnesium (38). Calcium and magnesium possibly share and compete for a common absorptive mechanism (39). The conflicting results are due to differences in amounts and the relative ratios between calcium, magnesium and phosphorus used in the various studies (38).

Many factors affect the solubility of calcium salts or the formation of complexes which may have different permeability to the gut wall. The effects of lactose, some other carbohydrate, amino acids, bile, bile salts and many organic acids on intestinal calcium absorption are considered to occur on the change of solubility and/or permeability of calcium salts.

Metabolic inhibitors such as fluoride, fluoroacetate, 2,4-dinitrophenol, phloridzin, and malonic acid increase calcium absorption <u>in vivo</u> and <u>in vitro</u> (7). Increase in absorption also might be due to the inhibition of oxidative metabolism which Schachter et al. (40) considered it necessary to maintain the relative impermeability of the

gut to calcium. Lactose, which inhibits oxygen uptake, phosphate uptake and the energy-dependent reactions, has also been considered by some investigators to enhance intestinal calcium absorption by this mechanism (41).

The efficiency of calcium absorption seems to depend in part at least, upon the level of calcium intake (Table 1). The adaptive mechanism and overall regulatory mechanism of calcium absorption are still not well understood (see page 25). Analysing the results of the kinetics of calcium metabolism in man and animal, Bronner et al. (30), Aubert et al. (42), Phang et al. (32), and Malm (43) found a linear relation between calcium absorption and other parameters of calcium metabolism such as urinary calcium excretion and rate of calcium entering bone. The intestinal absorption of calcium, therefore can either influence or be influenced by other parameters involved in calcium metabolism.

Although parathyroid hormone has been shown to increase intestinal calcium absorption, there is no evidence that it is a direct effect (44, 45). Many other hormones have also been shown either *in vivo* or *in vitro* to affect intestinal calcium in different degree (5). Because other parameters of calcium metabolism are also affected, the effects of hormones on intestinal calcium absorption may be an indirect result of the effects on kidney and/or bone (5).

Excretion: Calcium is excreted mainly in the urine and the feces. The sweat calcium loss is generally considered very small and rarely included in balance and kinetic studies.

In a review of the literature, Leitch and Aitken (46) estimated at less than 20 mg per day the calcium loss in sweat in a temperate and comfortable environment. A few studies, however, show a large sweat calcium loss. The summary of the results of these studies is shown in Table 2. The amount varies greatly because of environmental condition and physical activity. Methodology also contributes to the differences. Estimated sweat calcium loss varies from 0 to 6.2 mg per hour and less than 10 to 72 mg per day under non-sweating condition (Table 2).

Individuals subjected to prolonged heat and strenous physical activity become acclimatized (49, 52). This can affect the total amount of sweat, the concentration of calcium in sweat and the total calcium loss. The amount of sweat declines to 10-80% of the initial rate in 6 hours (52). The concentration of calcium in sweat also drops dramatically from 5.26 to 0.4 mg per 100 ml sweat by the end of a few hours (49).

The effect of calcium intake on sweat calcium loss has not been well evaluated. There is no consistent relationship between calcium concentration in sweat and calcium intake (49). There is also no evidence that a com-

Table 2

Sweat Calcium Losses of Adults

Estimated Amount	Temperature	Physical Activity	Remarks	Reference
Not detectable			Insensible Perspiration	(47)
<20 mg/day			"	(46)
90 mg/day			Sweating at 800 mg/hr.	(46)
15.4±0.8 mg/day			Non-sweating condition	Footnote[5]
<10 mg/day	ca. 24°C	Sedentary	42 days' study "	6
21.3 mg/hr.	Temperate	Treadmill & bicycle	3 hours' study	(48)
8.1 mg/hr. 111 mg/day[a]	22.2°C	Ergometer 50 min. 6-8 Cal./min. 50 min. 3-4.5 Cal./min.		(48)
11.6 mg/hr. 137 mg/day[a]	29.4°C	"		(48)
20.2 mg/hr. 201 mg/day[a]	37.7°C	"		(48)
6.2 mg/hr.	27-28°C	Rest		(49)
20.2 mg/hr.	37-39°C	Rest		(49)
8.5 mg/hr.	36°C	Rest		(50)
25.6 mg/40 min.	ca. 25°C	Three periods of ergometer		(51)

[a] 3 mg/hr. was added to the rest of time for estimation of daily losses.

pensatory mechanism exists between urinary calcium excretion and sweat calcium loss.

Calcium excretion in the urine is not only the most important route for excretion but also plays a role in calcium homeostasis. Many factors affecting general calcium metabolism, or kidney function tends to affect urinary calcium excretion. Because of our poor understanding of the calcium transport system in tubular cells, the mechanisms of these factors are little known.

The non-protein bound calcium is filtered through the glomerulus. The calcium filtered load is the product of glomerular filtration rate (GFR) and filtrable concentration of calcium. Variation in filtered load by either one or both of these two variables affects the urinary calcium excretion (53).

Some hormones (53, 54), protein and other foods (55-57), saline loading (55, 58) and drugs (55,59) have been shown to affect GFR to different extents. The analysis of the dynamics of urinary calcium excretion must take into account the wide variation in GFR between individuals. This can be done most easily by relating the urinary calcium excretion to a fixed volume of glomerular filtrate. The normal range is 0.05 to 0.15 mg calcium per 100 ml glomerular filtrate (53).

The filtrable calcium concentration in plasma is generally stable. Percentage of protein-bound calcium

can be changed only slightly by some factors such as pH (60), infusion of mannitol (61), and changes in plasma proteins (62). A small percent of calcium exists as a complex with anions (54, 63). Because these complexes are filtrable through the glomerular membrane, administration of anions such as citrate (62, 63), phosphate (61, 64), sulfate (64, 65) and EDTA (61, 66) is able to modify the concentration of filtrable calcium.

If GFR is 125 ml per min. and 60% of the calcium is filtrable, more than 11 gm of calcium is filtered per day. More than 98% of the filtered calcium is reabsorbed. In normal individuals, the filtered load is linearly correlated to the urinary calcium (53). However, calcium is always present in the urine even when the filtered load is very low (53). This may be due to the inability of tubular cells to lower the calcium concentration in lumenal fluid below a minimal level (about 5 mg% in rat, 67). A very high filtered load does not seem to saturate the reabsorptive mechanism (53).

The calcium is reabsorbed throughout the nephron with the proximal tubules as the major site. Two-thirds of the filtered calcium is reabsorbed in the proximal tubules, 20-25% in Henle's loop and 10% in the distal tubules. Diffusion seems to be the main process for calcium reabsorption especially when filtered load is increased. Active transport seems to take place along

the tubule but more in the distal tubule (67, 68). It has been proven that there is bidirectional tubular flux of calcium both *in vivo* and *in vitro* studies (54, 67, 69). The filtered calcium complexes with anions are reabsorbed either as complexes or as free ions after dissociation (53, 70).

An increase or decrease in the removal of water in the tubular lumen will change the concentration of calcium, therefore, any osmotic change induced by any factor is likely to affect the calcium reabsorption in tubules. Mannitol, sucrose, urea, saline have been shown to induce osmotic diuresis and subsquently enhance calcium excretion in urine (5, 53, 54, 71 for rev.).

The pronounced correlation between urinary excretion of calcium and other divalent alkaline earth cations has been observed for years (54). The highest degree of interdependence is found between calcium and strontium (54, 72). Still pronounced but a lesser correlation exists in decreasing order between calcium and magnesium, barium and radium (54, 73). A common transport mechanism for all these alkaline earth cations seems likely and this system transports calcium more readily than other cations (54, 73). However, this interdependence can be altered by dietary management such as low intake of magnesium (74), very high or low protein intake[1,2] or infusion of either calcium or magnesium[7]. Other mechanisms, therefore,

besides this common transport system may also exist.

Calcium excretion is also directly related to the excretion of sodium (53, 54, 71). The interdependence is attributed to tubular reabsorption of these two ions rather than the amount of filtered load (75). In vitro studies also show that the fractions of two ions which are reabsorbed are the same throughout the nephron (67, 68) indicating a common transport mechanism. Duarte and Waltson (76), however, suggested a coupling mechanism between these two ions rather than a common reabsorption system.

Hemodynamic factors also influence profoundly the urinary calcium excretion. Expansion of extracellular space (77-79), vasodilation by acetylcholine or bradykinine (80, 81), increase in mean arterial pressure by carotid occlusion or vagotomy (81) increase urinary calcium excretion. The vasodilation and elevation of mean arterial pressure have a synergic effect (81). Decrease in the arterial pressure, on the contrary, has the reverse effect (81). These hemodynamic factors do not affect the calcium filtered load (81). The site of mechanism of these hemodynamic factors must be beyond the glomerulus. Some investigators considered the inhibition of proximal tubular reabsorption to be responsible for this effect[8,9] (81, 82). The inhibition was thought to be due to the decrease in uptake of peritubular intersti-

tial fluid because of the hemodynamic alteration (81, 82). The effect on the other part of the nephrons, however, is unknown.

If the metabolism of tubular cells is modified, the reabsorption of calcium may be altered. Intake of glucose, fructose, galactose, amino acids, protein and some other nutrients (57, 83, 84, and Table 3), has been shown to increase urinary calcium excretion. Ingestion of alcohol (85) and infusion of insulin (83) have the same effect. Lindeman (83) attributed this effect to an enhanced glucose uptake and glycolysis in the tubular cells.

Metabolic acidosis and administration of acid can increase urinary calcium (54, 86), however, metabolic alkalosis and administration of alkali produced variable responses in urinary calcium excretion (54, 87, 88). These changes in urinary calcium have been attributed to the effect of pH on the metabolism of renal tubular cells (86), on calcium filtered load in glomerulus (88), on sodium clearance (75), or on bone minerals (89,90).

At least six hormones have been shown to affect the urinary calcium excretion. They are parathyroid hormone, calcitonin, thyroid hormone, gonadal hormones, corticosteroid hormones and growth hormone. The effects of these hormones have been reviewed by Bronner (5), Walser (54), Epstein (71) and Nordin et al. (53). The effects of these hormones have been considered to be either primary on

renal handling of calcium excretion or secondary to the effects of hormones on the bone and/or the gut. Different or contradictory results between investigators have also been reported frequently.

There are also other physiological, environmental and pathological factors influencing urinary calcium excretion. Diurnal and seasonal variation has been observed for years (53, 54). Differences in food and activity can affect this variation but hardly explain it (53). Activity, posture, weightlessness, temperature all have been shown to affect urinary calcium excretion in varying degrees (5, 53, 54, 71).

Calcium metabolism in bone and calcium homeostasis: Bone contains 99% of the total calcium in the body and the calcium which constitutes about one-quarter by weight of fat free dried bone, is the most important structural element in bone. The metabolism of either bone or calcium is influenced profoundly by the metabolism of the other.

Bone is constantly being replaced by resorption of existing areas and apposition of new bone. Osteoblasts secret soluble collagen into the extracellular space. The collagen then aggregates to form fibrils which comprise 95% of the organic substance of bone matrix. The rest consists mainly of mucopolysaccharide, glycoprotein, and phospholipid (91, for rev.).

A normal metabolism of organic matrix is essential

for a normal metabolism of calcium in bone. Metabolism of the main component of organic matrix, collagen, has been known for years to be affected by many dietary factors such as protein and essential amino acids (92, 93), iron (93), copper (93,94), vitamins C, D, A, E, B_6 and nicotinic acid (93,95). The physical structure of collagen may also be modified by these factors (91, 96). Many hormones such as growth hormone, thyroid hormone, parathyroid hormone and glucocorticoids can also alter the rates of synthesis and/or degradation of collagen (92).

The metabolism of bone matrix can be evaluated by plasma alkaline phosphatase activity and urinary hydroxyproline excretion. Plasma alkaline phosphatase originates primarily from osteoblasts and chondroblasts and small fraction from the liver cells (97, 98). The plasma activity of this enzyme, in the absence of liver disease, is correlated with bone matrix formation (99). Urinary hydroxyproline comes exclusively from breakdown of collagen. Collagen in bone consistutes more than half of total collagen in the body and is also 2-5 times more metabolically active than other collagen (100, 101). Urinary hydroxyproline, therefore, is a good index of degradadation of collagen in bone, if the dietary collagen can be limited (102, 103).

Calcification is initiated in the aggregated collagen fibrils. The exact mechanisms and processes of cal-

cification are still not clear. The unique structure of bone collagen seems to be essential for calcification (91 for rev.).

The resorption of bone takes place by dissolving the minerals and digesting the organic matrix. Enzymes secreted by the osteoclasts are thought to be responsible for resorption. To a lesser extent osteocytes can also resorb perilacunar bone (104, 105, 106 for rev.).

This continuous remodeling of bone is not only important for the structural integrity of bone but also plays a critical role in calcium homeostasis. Calcium is released from or taken up by bone during the remodeling. Increase or decrease of calcium in the bone is not only affected by the bone volume changes (apposition or resorption) but also by the continuous changing of calcium density in the bone (18).

Processes such as bone volume changes (apposition or resorption rates) or calcium density changes (augumentation or dimunition rates) can be measured quantitatively by various methods (104, 105, 18 for rev.). Internal marking with substance such as tetracycline (107) can measure the linear apposition rate. A rate of 0.8 ± 0.3 μ per day has been observed in the adult human rib (108). A more quantitatively and specific study of calcium dynamics in the bone can be achieved by using quantitative autoradiography (109). Calcium density can be calculated

from quantitative microradiography (110). Cameron and Sorenson (111) introduced the technic of photon bean scanning by employing ^{125}I to provide a monochromic energy source to measure bone mineral content.

All parameters using the above methods serve only to measure localized bone. Calcium metabolism in the bone of whole body is best studied by radiocalcium. This method was first developed by Bauer et al. (112, 113), Heaney and Whedon (114) and Aubert and Milhaud (115). They measured primarily the pool size and turnover rate. The rate of calcium entering and leaving bone can then be calculated from the pool size, turnover rate and calcium balance. These calculated rates represent not only a single physiological process but rather the result of multiple processes (104, 107). For example, the rate of calcium entering bone includes primary and secondary calcification, periosteocytic deposition, and long term calcium exchange.

The conventional method of radiocalcium technic is to administer single intravenous dose. Some also administer radiocalcium by continuous intravenous infusion (116) or continuous oral feeding (117). The measured values differ greatly between investigators due to differences in duration of study, methods of administration of tracer, theoretical basis and assumption for calculation and so on. Heaney (118), Aubert et al. (17) and

Marshall (18) have excellent reviews discussing these differences and the evaluation of these values.

The mechanisms controling the magnitude, location and balances of bone remodeling are still not clear (104, 105). One of these control mechanisms, parathormone-calcitonin mediated resorption is specially important for calcium homeostasis.

The plasma calcium level is strikingly constant. Wide variation in dietary calcium and calcium excretion produces no significant change in plasma calcium level. Diurnal fluctuation is less than 3% (119). The recovery from hypercalcemia induced by calcium infusion or hypocalcemia induced by EDTA injection is remarkably rapid in normal individuals (120 for rev.).

Parathyroid hormone (PTH) and calcitonin are continuously secreted to maintain normal plasma calcium level. Their rates of secretion are controlled by the the plasma calcium level. PTH secretion is increased by low plasma calcium. At least two types of metabolic effects on bone have been shown by PTH (121). One is an effect on osteocytes enhancing osteolytic activity and thereby releasing calcium rapidly. The other is to stimulate mesenchymal proliferation and osteoclast induction. Vitamin D seems to be required for these effects of PTH (104, 121). These effects of PTH also tends to be enhanced by heparin (122), low calcium and phosphate levels

(104, 123), but is inhibited by vitamin D deficiency (104, 123) and fluoride (124). Recent studies indicate that direct activation of adenyl cyclase is the primary action of PTH. The cyclic 3',5'-AMP is the intracellular mediator of the physiological action of PTH. (125 for rev.).

Calcitonin secretion, on other hand, is increased by high calcium plasma level (120). Calcitonin inhibits bone resorptive process. Bone formation is also shown to be increased by calcitonin. By these actions, calcitonin can inhibit the release of calcium from bone and lower the plasma calcium level (104, 105, 120 for rev.). The action of calcitonin seems not related to the adenyl cyclase-cyclic AMP system (125).

Besides these principle regulatory mechanisms, calcium homeostasis is also achieved to a lesser extent by the buffering action of a miscible pool, urinary excretion, intestinal absorption and excretion, and the effect of phosphate on the deposition of calcium in the bone and tissues (120).

Adaptation to low calcium intake

Extensive studies attempting to estimate calcium requirement have been done since the begining of this century (126 for rev.). The estimated calcium requirement for the maintainance of an adult man varies from 126 mg to 1020 mg per day or 1.9 to 15.7 mg per kg per day (126). A strikingly low calcium requirement among

the Chinese (127), Japanese (128), Peruvians (129), African Bantu (130) and Ceylonese (131) was noted regardless of the method of measurement used.

Most studies in Europe and U.S., however, show a remarkably negative balance with low calcium intake. A marked difference in calcium requirement is later observed in all populations regardless of race and geographical area (126). Low requirement seems to reflect mainly the past dietary habits and calcium reserve in the body (126).

Bauer et al. (132) did not observe a definite adaptive process in their long term experiment attempting to measure endogenous calcium loss. McClellan et al. (133) gave two arctic explorers a pure animal meat diet which usually supplied only 0.05-0.15 gm calcium per day. One subject showed a significant decrease in his calcium excretion about 90 days after the start of experiment. Thorangkul et al. (134) conditioned 2 groups of young men to different calcium intake levels, one group 350-750 mg per day and the other 1250-2000 mg per day. They then took part in a 16-day balance study with a low calcium intake of 175-253 mg per day. Both groups were in negative balance, but the group accustomed to a lower calcium intake excreted much less urinary calcium and had a lesser negative balance. During a 95-day extension of the study, both groups showed a gradual improvement in calcium re-

tention. More extentive study by Malm (126) using a longer period (average 240 days) and a larger group (26 men) showed marked individual differences in adaptive processes. After an average of 218 days on about 940 mg calcium per day intake, subjects received about 460 mg calcium per day for various periods (70-812 days). There were three different kinds of responses among the subjects:

1. Three subjects adapted immediately.
2. Improvement of negative balance after various periods (42-252 days) was noted for 20 subjects.
3. Three subjects showed a continuous negative balance up to 18 months after the experiment.

These adaptive processes have also been demonstrated in the rat (135), the dog (136) and cattle (137). Gershoff et al. (136) demonstrated that the calcium needed to maintain balance in adult dogs was largely a reflection of their previous calcium intake during growth. Age seems to retard the adaptive processes in the rat (135), but this fact is not well documented for human.

The adaptive processes are due to an increase in absorption and/or a decrease in urinary calcium excretion. Malm (126) indicated that intestinal adaptation was the most important factor. Thorangkul et al. (134) and Johnston and Flosom (138), however, showed that a decrease in the urinary excretion played a more important role in adaptation to low calcium intake.

The mechanisms that detect the sufficiency of calcium in the body and initiate a greater intestinal absorption and/or lesser urinary excretion are still unclear. Parathyroid hormone was suggested by some investigators as the regulatory factor for adaptation (7, 32, 139). Phang et al. (32) even suggested that intestinal absorption might act as a triggering mechanism for the secretion of the hormone. However, parathyroid gland and parathyroid hormone have been shown to be non-essential in the adaptive processes in animal studies (45, 140).

Nicolaysen (141) postulated an endogenous factor or factors produced in the bone in response to the unsaturation of bone to regulate intestinal absorption. Efforts to isolate these factors have not been successful. Stanburry (142) also indicated in his study that there was evidence of some humoral agents which regulated absorption according to prevailing needs of body. However, the study of Malm (126) showed that osteoporosis did not necessarily contribute to an efficient adaptation to low calcium intake.

The specific calcium binding protein of Wasserman et al. (11, 12) may play an essential role in these adaptive processes. This protein is increased in the intestinal mucosa of the animal adapted to a low calcium intake (143). The mechanism that triggers the increase of calcium binding protein is still unknown.

Adaptation to Dietary protein level

Protein is required by the body to provide essential amino acids and nitrogen for synthesizing amino acids, protein and other nitrogen containing substances. Other than the essential amino acids, nitrogen can be provided fairly well from non-protein or non-amino-acid nitrogen (144, 145). However, protein is still the most conventional source of both essential amino acid and nitrogen.

The gastrointestinal tract and digestive glands play an important role during alteration of the dietary protein level. Proteolytic enzymes such as trypsin in the digestive juice increase as dietary protein increase (146), however, protein hydrolysate does not induce this effect (147). Either nervous reflexes or hormonal agent may be involved in the regulation of the release of these enzymes. The gut is not a limiting factor for digestion and absorption even if dietary protein level is increased to 600 gm per day[10].

Ingestion of a meal stimulates the release of a large quantity of endogenous protein into the gut lumen. These endogenous proteins come from shed mucosal cells, digestive juices and plasma protein. Hydrolysis of this protein mixture yields an amino acid pool with a relatively constant ratio regardless of the meal ingested. This kind of amino acid regulation in the gut lumen can prevent the large fluctuation of the amount and the ratio of the amino

acid pool. This may contribute to a maximal efficiency in absorption and utilization of amino acids (148 for rev.).

With a lack of dietary protein or specific amino acids the gut and digestive glands decrease their protein content rapidly (149). However, it has been shown that there is a rapid restoration when the dietary protein level is increased (149). Liver, kidney, heart, skin, muscle and other tissues have also been shown to lose or restore the protein contents similarly but at different rates when the protein intake is varied. This concept of labile-protein or protein-reserve was introduced more than one century ago as one of the mechanisms that allows the body to adapt to variable protein intake (149-152 for rev.).

Another process, alteration of protein turnover, is also an important mechanism for adaptation. Turnover is meant to imply the replacement of an amount of degraded protein by an equal quantity of newly synthesized protein from its metabolic precursors or transported into the system from outside (153, 154). An abrupt change of either rate of synthesis or breakdown of protein can be induced by high or low protein intake. Modification in the rate of other process is followed until a new steady state is reached.

High turnover rate of many proteins has been found

during higher protein intake (152-154). This higher turnover is accompanied by increase in many enzymes involved in protein synthesis or degradation such as urea cycle enzymes (154, 155), enzymes for amino acid degradation and transamination (154-158). An excessive intake of a single amino acid also leads to an adaptative increase in degradation pathways of this amino acid (154, 159, 160). The presence of labile-protein or reserve-protein at least partly can be attributed to the changes of these "induced" enzymes.

Protein intake and calcium metabolism

An effect of dietary protein on calcium metabolism has been shown for a long time. Earlier in this century, calcium was considered to be absorbed more readily in the form of "calcium proteinate" than in other form (161 for rev.). Sherman (1) found that addition of meat to the diet caused an increase in urinary calcium. Mellanby (162) also observed a definite antirachitic effect in lean meat which was not comparable with that of antirachitic vitamins. All of these observation suggested that protein intake played a role in calcium metabolism. On the other hand, calcium in the diet was also shown to affect protein metabolism. Ranganathan and Rau (163) found the biological value and digestibility of protein in the Indian diet increased significantly in the rat after supplementation of calcium.

The observation of Sherman (1) was later noted repeatedly by Kunerth and Pittman (164), Pittman and Kunerth (165), McClellan et al. (133), and Hegsted et al. (129). The increase in protein intake after addition of meat to the diet was considered as the primary factor responsible for this calciuretic effect. McCance (2) further proved that the calciuretic effect could be induced by supplementation of partially purified protein source such as peptone, gluten, gelatin and egg white. The summary of the results of these studies is shown in Table 3.

There were only few subjects studied in most of these studies. Other than calcium intake, many other components of diet were not balanced at the two levels of protein intake. Because many dietary factors other than protein have been shown to affect the urinary calcium excretion greatly (see page 13-18), the significance of these studies, therefore, is obscured for not controlling the other dietary factors.

A series of experiments to study the metabolic response to varying protein intakes has been carried out by Calloway and Margen[12,13]. A more purified protein was used and dietary components other than protein were also carefully controlled to maintain a constant level. A direct relationship between urinary calcium and dietary protein level was noted[1,2,3]. A subject may excrete more than 8 times the amount of calcium during the highest

dietary protein level (about 600 gm protein per day) than he does during the protein-free diet period.

Fecal calcium is also found to be decreased in most of the studies during the higher dietary protein level (Table 3). Increase in urinary calcium excretion has been attributed by some investigators to an enhancement in intestinal calcium absorption[3] (2). The effect of protein intake on calcium retention, however, is not clear. Higher protein intake may cause an increase, decrease or no significant change in calcium retention Table 3).

Skeletal growth and structure are also profoundly affected by the quantity or quality of dietary protein. In animal studies dietary protein deficiency or lack of essential amino acids will slow down skeletal growth and alter skeletal structure (168, 169). The epiphyseal cartilage is narrowed, the number of the cartilage cells is reduced and the arrangement of cells is disturbed, and the trabeculae of the subepiphyseal zone is also decreased.

On the other hand, addition of extra protein to the diet also has a deleterious effect in bone. A diet low in calcium but high in protein can lead to a severe osteoporosis (169). Engstrom and DeLuca (170) also showed a lesser calcium content of bone in rats fed a high protein diet with either a low-phosphorus or low-phosphours-low-calcium intake than in the rats fed a medium intake of

Table 3. Effects of Protein Intake on Calcium Metabolism

Supplement of Protein		Calcium Intake		Urinary Calcium		Fecal Calcium		Calcium Balance		Number of	Reference
Type	Amount g/day	B[a]	A	B	A	B	A	B	A	Subjects	
		mg/day									
Meat	?	390	400	280	360	110	190	0	-150	1	(1)
Milk	25	448	452	99	106	513	326	-164	+26	3	(166)
Soy bean curd	24	448	451	111	115	443	322	-106	+14	3	(166)
Meat	35	458	436	123	136	416	334	- 81	-34	3	(164, 165)
Peptone, egg white, gluten, gelatin	100-130	634	625	72	107	602	531	-40	-13	3	(2)
Meat	36	362	338	129	144	214	165	+19	+29	10	(129)
Meat, milk	93	1408	1407	175	338	1223	1154	+10	-84	6	(167)
Egg albumin	73	900	900	107	190	--	--	--	--	4	Footnote[2]
Egg albumin, soy protein, casein	525	2300	2300	100%	438%	--	--	--	--	4	" [3]

[a] B, before and A, after supplementation of protein

protein with the same level of calcium and phosphorus. Increase in calcium intake in these two experiments can prevent this effect. In two weight-controlled groups of rats fed at different levels of casein, Shenolikar and Narasigna Rao (171) showed that the group maintained at the high dietary protein level (casein 40%) had a significant decrease in ash content of the bone as compared to that of the lower dietary protein level (5%). The calcium intake in this experiment, however, was not low (0.5% in the diet).

To date the mechanisms of these effects of protein intake on calcium metabolism have not been well studied. Amino acids and urea have been shown to increase the solubility of calcium salt (172, 173) and/or to form complex with calcium (173, 174). Wasserman et al. (175) demonstrated that lysine and arginine promote the intestinal absorption of calcium and other amino acids have little or no effect. Raven et al. (176) also proved that lysine had to be present with calcium in order to enhance calcium absorption in a ligated intestinal segment. The theory that amino acids increase the solubility or formation of complexes does not seem to explain the differences between the amino acids. Many amino acids have a pronounced solvent action or form stable complexes but have little or no effect on calcium absorption (174).

Variable protein intake can exert some changes in

renal function. The role of these changes in calciuretic effect of high protein intake has not been well evaluated.

Endogenous creatinine clearance is a good and also convenient method to evaluate glomerular filtration rate (GFR, 56, 177). Variable protein intake has been shown to affect the creatinine clearance rate (177 for rev.). Because tubular secretion of creatinine exists (178), it is not certain the increase in creatinine clearance during higher protein purely results from increase in GFR. However, a consistent relationship between inulin clearance rate and protein intake strongly indicates that GFR is increased by dietary protein (179). The mechanism by which protein feeding increases GFR is still not well known. GFR in the dog is shown not only to be increased immediately after protein feeding but also persists for hours or days (180). The action of protein is probably due to the effect of amino acids, because feeding or infusion of amino acids has a similar effect (181)

Other than GFR, the enzymatic activity involved in transport has also been shown to be modified by variable protein intake. Katz and Epstein (182) fed rats a high protein diet (50% protein) for 7 days. The specific activity of sodium-potassium-activated-adenosine-triphosphatase (Na-K-ATPase) in the kidney was increased significantly. Other enzymes such as glucose-6-phosphatase, glutaminase or succinic dehydrogenase were not affected.

Na-K-ATPase is directly involved in the active transport system for sodium. The increase in activity of enzyme is correlated to the increase in reabsorption of sodium. However, no comparable research has been done to study the relationship between enzymes involved in calcium reabsorption and protein intake.

The mechanism of the effect of high protein intake on bone has also not been well studied. Engstrom and DeLuca (170) considered some unknown factor in egg white to be responsible for decrease in bone mineral content in their study. This factor is associated with conalbumin-ovomucoid-lysozyme fraction of egg white, but the nature of such a factor is unknown. However, El-Maraghi et al. (169) using semi-synthetic diets containing mainly casein, maize, starch, cellulose and other supplements still noted a similar effect. They considered that a high protein diet promoted the deposition of bone matrix. When there was insufficient calcium intake, the resorption of existing bone to provide minerals for the new area was taken place probably through stimulation of the parathyroid hormone. This mechanism is postulated to lead to the development of osteoporosis.

Decrease in food intake has been shown to block collagen cross-linking in rats (96). Structural changes of collagen were also noted by McClain[14] when the animal was fed various diets (including high protein diet). These

observations were found in collagen in skin and connective tissue other than bone. Because the collagen in the bone is 2-5 times more metabolically active than the other collagen (100, 101), the structural changes in bone collagen are very likely to be even more responsive to the changes in protein intake. The unique structure of bone collage is essential for normal calcification (91). The possible structural changes of bone collagen by high protein intake may be an important mechanism leading to the development of osteoporosis.

Prolonged acid loading has been shown to cause an increase in urinary calcium excretion and bone resorption (54, 86, 89, 90). It is also well known that protein produces an acid ash. Therefore, some investigators considered that the effects of dietary protein on calcium metabolism were mainly those of acid loading (90, 183). Ingestion of bicarbonate at the same time as protein supplementation could reduce substantially the calciuretic effect of high protein intake (183). The exact mechanisms by which prolonged acid loading affects calcium metabolism are still unclear (see page 17).

EXPERIMENTAL

Six healthy male volunteers were confined to a metabolic unit for sixty days. These subjects ranged in age from 22 to 32 years. Their weights and heights on admission ranged between57.5 to 82.4 kg and 172 to 192 cm, respectively (Table 4). The study was divided into four metabolic periods of 15 days each. During each of the first three periods the subjects received a low calcium diet (ca. 100 mg per day) with one of the following levels of protein: protein-free (0.9 gm nitrogen per day), medium-protein (12 gm), high-protein (24gm). The order in which the diets were given varied for every subject (Table 5). During the last period all subjects received the medium-protein diet but four subjects were supplemented with approximately 900 mg calcium per day. They were all ambulatory. Exercise periods were required every day which consisted of two 30 minutes sessions walking on a treadmill set at 3 miles per hour at a 10% grade. Other exercise was not controlled and additional activities were allowed.

Diet composition, preparation and analysis:

The diets contained the smallest amount of calcium that could be obtained at a protein level of 24 gm nitrogen per day. In order to prepare such low calcium diets with three different levels of protein, individual dietary components were analyzed and calculations were

Table 4.

Description of Subjects

Subject	Age (yrs)	Weight (kg)	Height (cm)	Surface Area[a] (m^2)
1	22	82.4	192	1.95
2	22	80.4	182	1.86
3	25	57.7	172	1.55
4	32	63.9	180	1.68
5	23	66.2	181	1.71
6	24	71.7	183	1.77

[a]According to Boothby-Sandiform Nomogram

Table 5.

Dietary Periods Design[a]

Order of Period	Subject					
	1	2	3	4	5	6
1	24N	0.9N	0.9N	24N	12N	12N
2	0.9N	24N	12N	12N	24N	0.9N
3	12N	12N	24N	0.9N	0.9N	24N
4	12N	12N	12N+Ca	12N+Ca	12N+Ca	12N+Ca

[a]Diets: 24N: high-protein diet, 24 gm nitrogen per day
12N: medium-protein diet, 12 gm nitrogen per day
0.9N: protein-free diet, 0.9 gm nitrogen per day
+Ca: calcium supplementation, 900 mg per day

made for various combinations of food and formula. Three diets were then developed which contained approximately the same food composition except for the amount of protein and carbohydrate. The composition of three diets are shown in Table 6.

Samples of the diets, both individual food items and total composites were well homogenized using a blender and a Polytron (Linematica GMBH, Luzern-Schweiz). Nitrogen was determined by micro-Kjeldahl method. After wet ashing with a perchloric-nitric-sulfuric acid mixture, the content of sodium, potassium, calcium and magnesium was determined by atomic absorption spectrophotometry (184), phosphorus by an automated adaptation of the phosphomolybdic reduction method (185). Aminco-Cotlove Chloride Titrator (American Instrument Co.) was used for chloride determination (186). The variation in dietary intake of nitrogen and minerals is shown in Table 7.

All the food and formula were prepared in large quantities, weighed into containers for individual meals, and frozen until needed. Diets were then defrosted, heated if required, and served. Each subject received four meals a day at 8:30 A.M., 12:30P.M., 5:30 P.M. and 9:30 P.M.. Table 8 indicates the menus and methods of preparation of some food items for the various meals during each period. The caloric intake needed by individual subjects to maintain body weight was provided by

Table 6 Diets Composition

Items	Brand Name	Diets 0.9N	12N	24N
		gm / day		
Dextrimaltose	Mead Johnson	100	--	--
Cornstarch	Buffalo	90	20	15
Lard	Wilson	34	--	--
Margarine, salt free	Safeway	34	38	30
Safflower oil[a]	Co-Op	15	12[b]	20[b]
Citrus Pectin	Sunkist Growers	1.0	0.64	0.60
Sucrose	C & H Cane	75	--	--
NaCl	c	4.0	3.0[b]	2.0[b]
Rusk, low protein	Carlo Erba, Milan Italy	130	100	64
Spaghetti, low protein	Carlo Erba	--	100	50
Minute Rice	General Food	--	35	35
Corn oil	Mazola oil	--	10	--
Turkey, all white	Armour	--	140	316
Cranberry sauce	Ocean Spray	--	90	100
Beef, ground & low fat	CO-Op	--	182	355
Soysauce	Kikoman, Japan	--	5[b]	5[b]
Peanut oil[a]	Planters	--	10[b]	--
Olive oil[a]	Star	--	--	8.5[b]
Dry banana	Beatrice Foods	30	30	--
Lecithin	Midland	31	31	--
Magnesium Oxide	c	0.95[f]	0.87[f]	0.75
Ca Carbonate	c	0.077[f]	0.03[f]	--
KH_2PO_4	c	5.0[d]	1.8[e]	--
KOH, 85%	c	0.07[d]	0.175[e]	--
NaOH, 97%	c	0.98[d]	0.45[e]	--
NaCl,	c	3.5[d]	1.3[e]	--
Benzoid acid	c	0.016[d]	0.002[e]	--
HCl, 11.4N	c	--	0.18 ml[e]	--
Tea, instant	Lipton	4.0	4.0	4.0
Vitamin Cap. A	h	1 cap./day	1cap./day	1 cap./day
" " B		"	"	"
Trace Min. Cap.	h	3 cap./day	3cap./day	3 cap./day
Choline Tabl.	h	--	--	4 Tab./day

[a] Different oils used in order to match the polyunsaturated fatty acids in three different diets.

[b] Components of meat sauce (see Table 8 footnote f)

Table 6 Diets Composition (continued)

[c]Reagent grade chemicals

[d]Minerals were dissolved in 40 ml water to make mineral solution 1 and 10 ml was given each meal.

[e]Minerals were dissolved in 40 ml water to make mineral solution 2 and 10 ml was given each meal.

[f]Magnesium oxide and calcium carbonate were mixed with dry banana, because it would form precipitate with other minerals in the mineral solution.

[g]For pH adjustment

[h]See Table 9

Table 7 Nitrogen and Minerals Contents of Composites[a]

Diet	Nitrogen	Calcium	Sodium	Potasium	Magnesium	Phosphorus	Chloride
				gm/day			
0.9N	0.87-0.91	0.097-0.100	3.45-3.47	3.04-3.16	0.64- 0.69	2.00-2.07	4.44-4.54
12N	11.9-12.2	0.105-0.106	3.03-3.08	3.12-3.44	0.62- 0.68	2.00-2.02	4.05-4.15
24N	23.4-24.2	0.086	3.02-3.19	2.67-2.78	0.59-0.62	1.64-1.69	4.12-4.31

[a]Composite samples, intake of an entire day. The part for caloric adjustment was not included.

Table 8 Menu of Different Diets[a]

Meal Time	Diet		
	Protein-free (0.9N)	Medium Protein (12N)	High Protein (24N)
8:30 A.M.	Tumbler of formula[b] Rusk & Margarine Lecithin Mineral solution 1 Instant tea	Rice[c] Rusk & Margarine[d] Turkey & Cranberry sauce Mineral solution 2 Instant tea	Rice Turkey & Cranberry sauce Instant tea MgO & Pectin solution
12:30 P.M.	Same as above plus Dry banana with MgO and $CaCO_3$	Spaghetti[e], beef & meat sauce[f] Mineral solution 2 Instant tea	Beef & meat source[f] Rusk & Margarine MgO & Pectin solution Instant tea
5:30 P.M.	Same as meal of 8:30	Same as meal of 8:30 except rice omitted	Same as meal of 8:30 except the rice omitted and rusk & margarine added
9:30 P.M.	Same as meal of 8:30	Rusk & margarine Spaghetti, beef & meat sauce Mineral solution 2 Instant tea	Rusk & margarine Spaghette, beef & meat sauce MgO & Pectin solution Instant tea

[a] Daily amount and characteristics of different food items shown in Table 6, equal parts were divided from this daily amount if served for more than once a day.

[b] Dry ingredients (dextrimaltose, cornstarch, citrus pectin, sucrose, salt) were first weighed and mixed. Correct amounts of lard and salt free margarine were melted and mixed with weighed safflower oil, then the fats were mixed with the dry ingredients. The formula were hydrated with deionized water and brought to a temperature of 78°C. After blending, the formula was weighed into individual tumblers and stored frozen until served.

[c] Rice was prepared by pouring boiling water into the rice containers with minute rice in it.

[d] Precooked frozen turkey was heated in foil at 350°F for 15-30 min.

[e] Spaghetti was boiled in deionized water for about 8 min. and served with beef and meat sauce.

[f] Weighed ground beef was browned. Meat sauce was made from the meat juices, water, cornstarch, soy sauce, salt and different oils (12N and 24N diets had different components, see Table 6). The beef and meat sauce were divided into individual containers frozen with the spaghetti until served.

Table 9 Content of Vitamin, Trace Minerals Capsule[a]

Vitamins	mg/capsule	Trace Minerals	mg/capsule
Capsule A			
Thiamine mononitrate	2.20	$FeSO_4 \cdot 7\ H_2O$	16.7
Riboflavin	3.15	$CuCl_2 \cdot 2\ H_2O$	1.79
Pyridoxine hydrochloride	5.25	$ZnSO_4 \cdot 7\ H_2O$	21.9
Vitamin B_{12} (1% resin adsorbate)	0.24	$MnSO_4 \cdot H_2O$	5.12
		$Na_2MoO_4 \cdot 2\ H_2O$	0.21
Biotin	0.06	$Cr_2(SO_4)_3 \cdot 15\ H_2O$	1.07
Vitamin K	1.20	$Na_2SeO_3 \cdot 10\ H_2O$	0.016
Ascorbic acid	55.0	$AlK(SO_4)_2 \cdot 12\ H_2O$	28.3
		KI	0.2
Capsule B			
Folic acid	0.66		
Niacinamide	21.0		
Calcium panthothenate	12.0		
Vitamin E	25 IU/capsule		
Vitamin A	4307 USP/capsule		
Vitamin D	440 USP/capsule		
Choline Tablet			
Choline dihydrogen citrate	0.65 gm/Tablet		

[a]Prepared for Department of Nutritional Sciences, University of California, Berkeley by Miles Laboratories, Elkhart, Indiana.

addition of different amounts of sugar and candy[15] to the basal diet. Vitamins and trace minerals were supplemented as capsules. The amount and composition of these capsules are shown in Table 6 and 9. In addition to the 1000ml tea per day, the subjects were required to drink 1000 ml distilled water. Additional water intake was unrestricted but the extra volume was recorded.

Subjects 3,4,5 and 6 received supplements of calcium gluconate during the last period (period 4). The calcium content of the calcium gluconate tablets (10 grain tablet, United Pharmaceutical Co.) was analyzed using the same method as for the diet. Sixteen tablets were weighed and four tablets were consumed during each meal time. The amount of daily calcium supplement was calculated from the weight of these tablets and the analyzed value for calcium content.

Sample collection and analysis

Urine:

Daily urine was collected and stored in the refrigerator without preservative. Urine weight, pH, specific gravity and osmolarity[16] were determined after each daily collection was completed. Rapid screening tests[17] were done for protein, glucose, and ketone bodies. Determinations for calcium, phosphorus and creatinine were made on 24-hour collections. Aliquots of daily urine samples collected for three days were combined and deter-

minations of the concentration of sodium, potassium, magnesium, nitrogen, and hydroxyproline were performed on these pooled samples. Creatinine was measured by an automated adaptation of alkaline picrate method (187) and hydroxyproline by a slightly modified method of Prockop and Udenfriend (188). Other methods of analyses were the same as those used for the wet-ashed aliquots of the diet.

Feces:

Fecal collection of first three days was not analyzed. During the remainder of the period, the feces were collected either daily (during ^{47}Calcium study) or in period of 2 to 6 days. Times of defecation and fecal weights were recorded. Daily or pooled samples were well homogenized with deionized water using a Gifford-Wood-Eppenbach Colloid Mill or polytron and aliquots frozen for further analysis. Total nitrogen, calcium, potassium, sodium, magnesium were analyzed using the same methods as for the diet.

Sweat:

Samples for measurement of sweat losses were collected by two methods. One represented the normal loss for ambulatory subjects while the other method measured the loss after strenous exercise. The method developed by Sirbu et al. (189) was used for samples collected between day 8 to 14 of each period. On the last day of each

period, sweat was collected after a short period of strenous exercise on a bicycle ergometer following the method described by Costa et al. (51). Change of room temperature was compensated by slight change in work load in order to collect roughly the same amount of sweat. All samples were analyzed for sodium, potassium, calcium, magnesium and nitrogen content by the same methods used for urine, feces and diets.

Blood:

Fasting venous blood samples were taken by venipuncture before breakfast on day 8 and at the end of each period. Creatinine, calcium, magnesium and phosphorus were analyzed without prior digestion by the same methods used for urine, feces or diet. Standard automated assays (190) were used for urea nitrogen and glucose. Alkaline phosphatase was determined by an automated method adapted from that of Bessey et al. (191). All constituents were analyzed on blood samples obtained at the end of each period but only calcium, phosphorus and alkaline phosphatase were determined on samples of day 8.

^{47}Calcium studies

^{47}Calcium chloride, containing less than 1% ^{45}calcium, was purchased from Amershan/Searl Corporation. An intravenous injection study was done during the second period and an oral ingestion study was performed during the third period.

The solution for injection was prepared by diluting the radioactive calcium chloride solution with pyrogen-free water and autoclaving. The solution containing approximately 1 μci radiocalcium was injected intravenously one to two hours after breakfast on day 7 of period 2. The radiocalcium solution was injected directly into infusion tubing while normal saline was flowing into the median cubital vein and then flushed with an additional 30-50 ml saline. The difference in the weight of the syringe before and after the injection was used to calculate the precise amount of radioactivity injected. Urine and feces were then collected daily for seven days for measurement of radioactivity.

The ^{47}calcium solution for oral ingestion was prepared by diluting the purchased solution with distilled water. From day 5 to 12 of the third period the subjects ingested equal amounts of radiocalcium solution (5.0 ml) four times a day at meal time. The cup which contained the radiocalcium was rinsed several times with drinking water and these solutions were also consumed. The dilution of radiocalcium solution was calculated so that the amount of radioactivity ingested was about 0.5 μci per day on the fourth day (i.e. day 9 of third period) of the ingestion period. Urine and feces were collected for a total of 14 days.

^{47}Calcium was counted in a well-type sodium iodide

scintillation counter with pulse height spectrometer analysis (model 410A Auto-Gamma Spectrometer, Packard Instrument Co.). Gamma emission of ^{47}scandium, the daughter element of ^{47}calcium, was excluded by the discriminator at a base level of about 400 kev. All samples were counted in the same kind of counting tube and counted with the same geometric conditions. Because of adherence of radiocalcium to the wall of the counting tube which could change the geometry of the counting condition and give higher counts, non-radioactive (cold) calcium chloride was added to the samples of the test solution for injection or ingestion in order to minimize this effect. Since cold calcium was already present in the urine and fecal samples, calcium chloride was not added to these samples. All counts were then adjusted to zero time.

Because of the very low level of radioactivity in the urine and feces they were not counted directly. Urine was first acidified and heated. Oxalic acid was added and the solution then neutralized to pH 5 with concentrated ammonium hydroxide. The precipitate was dissolved in concentrated hydrochloric acid. This solution was used for measurement of radioactivity and calcium content. The feces were also acidified and homogenized. A volume of acetone equal to twice the fecal homogenate was added to facilitate filtration and the

entire slurry was filtered. Calcium was then precipitated from the filtrate by the same method used for the urine. The slurry precipitate was transfered to the counting tube for radioactivity determination[18]. After radioactivity was determined the slurry was dissolved in concentrated hydrochloric acid and filtered. Calcium content was then determined. During the oral ^{47}calcium study, slurry precipitate was dissolved in concentrated hydrochloric acid. The supernatant solution obtained after centrifugation was transferred to the counting tube for counting and later for determination of calcium. From the specific activity and the amount of calcium in the daily urine and feces, the amount of radioactivity in the daily urine and feces was calculated. The detailed procedures of preparation of urinary and fecal samples for radioactivity determination can be found in Appendix 1 and 2.

The model and theory used for calculation of quantification of calcium metabolism was based on that of Aubert et al. (17) and Bronner (5). The definition and notation of these rate processes were similar to those of Aubert et al. (17). Table 10 lists the definition of these notations.

Endogenous fecal calcium was calculated from the following formula for the intravenous ^{47}calcium study.

$$V_{ef} = \frac{R_F}{R_u} \times V_u \qquad \text{-------- Equation 1}$$

Table 10 Notation and Definition

	Definition
V	Rate process of calcium metabolism, in mg/day
V_i	Calcium ingested
V_a	Ingested calcium that is absorbed(Ca absorbed)
V_d	Calcium secreted from the various digestive juices(Digestive juice calcium)
V_a'	Calcium absorbed from both ingested and digestive juice calcium
V_{ef}	Endogenous fecal calcium
V_s	Calcium loss in sweat
V_F	Total fecal calcium
V_u	Urinary calcium
V_o+	Calcium that enters the skeleton by a unidirectional process
V_o-	Calcium that leaves the skeleton by a unidirectional process
V_T	Calcium turnover, i.e. calcium lost from poolby V_u, V_s, V_{ef}, V_o+ , $V_T = V_u+V_{ef}+V_s+V_o+$
R	Rate process of radiocalcium, in cpm or % dose per day or other equivalent unit
R_{inj}	Radiocalcium injected
	The subscripts under R has the similar meaning as that under V
S.A.	Specific activity of radiocalcium in cpm/gm calcium or % dose/gm calcium
$S.A._{ecf}$	Specific activity of radiocalcium in extracellular fluid
	The subscripts under S.A. has the similar meaning as that under V
P	Miscible calcium pool size
Δ	Calcium balance
α	Fractional absorption rate of ingested calcium

The fractional absorption rate and digestive juice calcium was then defined as:

$$\alpha = \frac{V_i + V_{ef} - V_F}{V_i} \qquad \text{----------Equation 2}$$

$$V_d = \frac{V_{ef}}{1 - \alpha} \qquad \text{----------Equation 3}$$

The logarithm of the specific activity of radiocalcium in the urine was plotted against the time corresponding to the midpoints of the collection periods (Figure 2). The curve of disappearance of radioactivity in extracellular fluid is supposed to be linear between 24 to 144 hours after injection (5). The method of least squares was used to estimate the regression line, slope and the intercept by extrapolating the linear portion of the curve to zero time. The miscible pool was then calculated from the equation:

$$S.A._{ecf}^{o} = \frac{R_{inj}}{P} \qquad \text{------Equation 4}$$

where $S.A._{ecf}^{o}$ represents specific activity of extracellular fluid at zero time calculated from the extrapolating intercept at zero time, and R_{inj} was the injected radioactivity.

The slope m of regression line gives the turnover rate which is expressed as pool replaced per day, i.e. $m = V_T / P$. Because we assume that no labelled calcium returned to the pool from the bone and that the radiocal-

cium in the pool disappeared only through the urine, feces, sweat and entering into bone, the rate of calcium entering bone can be calculated by the equation:

$$V_{o+} = V_T - V_u - V_F - V_s \quad \text{--------Equation 5}$$

Since the calcium pool is assumed to be constant, the calcium balance can be accounted for by the amount of calcium entering or leaving bone. The rate of calcium leaving bone is then calculated from the equation:

$$V_{o-} = V_{o+} + \Delta \quad \text{----------Equation 6}$$

A technic of oral administration of radiocalcium similar to the one used in this study has not been reported previously in the literature. We adopted two methods for calculation of fractional absorption rate for our oral ^{47}calcium study. The assumption of Blau et al. (24) for calculation of total digestive juice calcium was used, although their formula was used for an intravenous radiocalcium study. The specific activity for urine on 5-7th day and for feces on the 6-8th day after starting the intake of radiocalcium was rather stabilized. The average specific activity of these intervals was used for calculation in the following formula:

$$V_d = \frac{R_i - S.A._F \times V_i}{S.A._F - S.A._u} \quad \text{---------Equation 7}$$

From the digestive juice calcium, endogenous fecal calcium and fractional absorption rate could be obtained

by the same definition used before.

Another method used by Aubert et al. (17) for calculation of endogenous fecal calcium after a single dose of radioactive calcium could be applied to our study. The endogenous fecal calcium was calculated by the following equation:

$$V_{ef} = \frac{V_u \times (R_i \times V_F - V_i \times R_F)}{R_i \times V_u - R_u \times V_i} \quad \text{---Equation 8}$$

The fractional absorption rate and the digestive juice calcium were then calculated as before.

The assumption and derivation of equation 1, 7 and 8 can be found in Appendix 3.

RESULTS

General

All the subjects remained healthy throughout the entire experiment except for subject 6 who had a mild cold during the fourth period (medium-protein diet plus calcium supplementation) and subject 3 who developed a small canker on the oral mucosa for a few days during the third period (high-protein diet period). There were no serious complaints throughout the experiment except that one subject complained of headaches and backaches occasionally and another subject complained of soreness of the tongue.

All the subject were able to consume all of their diets, although some complained that the protein-free formula diet was hard to eat. Every subject except subject 4 maintained his body weight within ± 1.5 kg of his initial weight on admission. Subject 4 gained weight amounting to a maximum of 2.7 kg. This gain may possibly have been compensatory for a weight loss he experienced a few days before admission. All subjects engaged in their daily activity throughout the experiment without any problem.

There was no serious problems with bowel movement. Four subjects had occasional loose stools during various periods. The bulk of feces was noted to be much less and the consistency harder in every subject during the high-

protein diet period. However, there was no constipation.

The weight of the urine ranged between 1600 and 2800 gm per day and there was no significant difference between periods. Specific gravity was between 1.003 and 1.018 and was directly related to protein intake. Urinary pH values tended to decrease as dietary protein increased; however, high calcium supplements were associated with more alkaline urine than during the period on the same diet without the calcium supplement. Osmolarity of the urine as well as the total excreted solute was increased during the higher protein intake periods. The mean total solute excreted during the period of high-protein intake was more than twice that during the protein-free diet period. There was no significant change in total solute excreted for subjects 3 and 4 when the calcium supplement was given; however, total solute excretion decreased greatly for subjects 5 and 6 when the calcium supplement was administered (Table 11).

The rapid screening test did not reveal protein or ketone bodies in the urine of any subject throughout the experiment. A positive glucose test was detected almost every day on the urine of subject 6 during periods 1, 2 and 3. The glucose became negative during the last period when the supplement of 900 mg calcium was given. A slightly positive urinary glucose test was also noted in subject 1 about half of the time during the high-protein

Table 11 Weight, Specific Gravity, Solutes and pH of Daily Urine[a]

Subject	Diet	Period Order	Urine Weight	Specific Gravity	Solutes Osmolarity	Solutes Total	pH
			gm/day		mOsm./L	mOsm./day	
1	0.9N	2	2101	1.007	278	680	6.58
	12N[b]	3	2001	1.010	362	711	6.58
	12N[b]	4	1747	1.012	427	682	6.47
	24N	1	1949	1.016	643	1221	6.35
2	0.9N	1	1824	1.007	270	484	6.46
	12N[b]	3	1777	1.011	385	669	6.26
	12N[b]	4	1707	1.011	395	639	6.22
	24N	2	2040	1.011	500	1022	6.19
3	0.9N	1	1904	1.007	250	473	6.55
	12N	2	1831	1.007	348	632	6.36
	24N	3	1876	1.013	577	1052	6.11
	12N+Ca	4	1605	1.010	404	631	6.57
4	0.9N	3	2114	1.006	220	457	6.69
	12N	2	2489	1.006	273	676	6.48
	24N	1	2787	1.008	386	1066	6.41
	12N+Ca	4	2155	1.007	286	619	6.73
5	0.9N	3	2452	1.006	195	485	6.70
	12N	1	2258	1.007	313	695	6.47
	24N	2	2437	1.009	454	1077	6.39
	12N+Ca	4	2128	1.008	278	571	6.68
6	0.9N	2	2059	1.006	245	499	6.58
	12N	1	1990	1.008	378	738	6.38
	24N	3	2183	1.010	427	966	6.48
	12N+Ca	4	1984	1.008	323	624	6.71
Mean	0.9N		2076	1.007	243	496	6.59
	12N		2057	1.008	343	687	6.42
			2142[c]	1.007[c]	328[c]	685[c]	6.42[c]
	24N		2213	1.010	498	1069	6.32
	12N+Ca		1968[c]	1.008[c]	323[c]	611[c]	6.67[c]

[a] Average of daily analysis but the result of first three days of each period were excluded.

[b] Diet was not changed between periods 3 and 4 for subject 1 and 2.

[c] Mean of subjects 3,4,5 and 6

diet period but not during the other periods.

Calcium metabolism

Even on the same dietary regimen there was wide variation in urinary calcium excretion between individuals. A magnitude of difference as great as 8 times was noted between 2 subjects during the same dietary period (Table 12). The daily variation for the same individual was also fairly large. Figure 1a-f show the dramatic and rapid changes during the first few days of each new period. The daily amount fluctuated randomly during most of the periods. There was a tendency towards a gradual decrease in urinary calcium excretion during the course of some dietary periods. This was especially true during period 3 and 4 when the diet remained the same for 30 days for subject 1 (Figure 1a). However, there were also certain periods in which the daily urinary calcium excretion increased gradually (Figure 1a period 2).

Every subject excreted more calcium in the urine, regardless of the order of periods, when the dietary protein intake was increased even though the calcium intake was low. They excreted an average of about 100 mg calcium in the urine per day during the medium-protein diet period. The amount was decreased about 50 mg when the protein-free diet was fed and increased to more than 160 mg per day during the high-protein period (Table 12). During the high-protein period, in all but subject 3, the average

Table 12 Calcium Intake, Excretion, and Balance

Subject	Diet	Period Order	Calcium Intake[a]	Urinary Calcium[b]		Fecal Calcium[c]		Sweat Calcium[d]		Calcium Balance	
				mg/day							
				mean ± S.D.							
1	0.9N	2	101	86	22	142		17		-144	
	12N	3	107	253	15	131		19		-296	
	12N[e]	4	107	208	14	137		19		-257	
	24N	1	88	258	14	185		36		-391	
2	0.9N	1	100	38	7	174		17		-129	
	12N	3	107	65	10	139		21		-118	
	12N	4	107	55	8	157		22		-127	
	24N	2	87	134	22	147		5		-199	
3	0.9N	1	98	10	3	198		14		-124	
	12N	2	105	33	5	162		10		-100	
	24N	3	85	65	14	130		17		-127	
	12N+Ca	4	1002	62	15	907		15		+ 18	
4	0.9N	3	98	33	10	194		11		-140	
	12N	2	105	59	13	125		11		- 90	
	24N	1	86	96	12	123		9		-142	
	12N+Ca	4	1003	163	13	950	?	12		-123	?
5	0.9N	3	101	58	19	144		15		-116	
	12N	1	107	65	17	114		14		- 86	
	24N	2	88	132	15	77		15		-138	
	12N+Ca	4	1005	211	20	766		13		+ 15	
6	0.9N	2	101	83	10	184		6		-172	
	12N	1	107	116	10	127		16		-152	
	24N	3	88	282	21	119		17		-330	
	12N+Ca	4	1066	205	26	723		16		+ 62	
				mean±S.D		mean±S.D.		mean±S.D.		mean ± S.D.	
Mean[f]	0.9N		100	51	30	174	25	13	4	-138	20
										(-136	22)[i]
	12N		106	99	80	133	16	15	4	-140	80
										(-109	27)[i]
				(68	34)[g]	(134	25)[h]	(13	3)[g]	(-113	35)[h]
	24N		87	161	88	128	37	17	11	-222	114
										(-187	86)[i]
	12N+Ca		1004	160	69[g]	799	96[h]	14	2[g]	+ 32	26[h]

[a] Based on the composite diet samples plus individual calorie adjustment

[b] Mean and S.D. of daily analysis but the results of first 3 days excluded

[c] Average of samples collected between day 4-15 of each period

[d] Sweat collected between day 8-14 of each period

[e] No dietary change between period 3 and 4 for subject 1 and 2

[f] Mean and S.D. of individual average of 6 subjects or otherwise specified

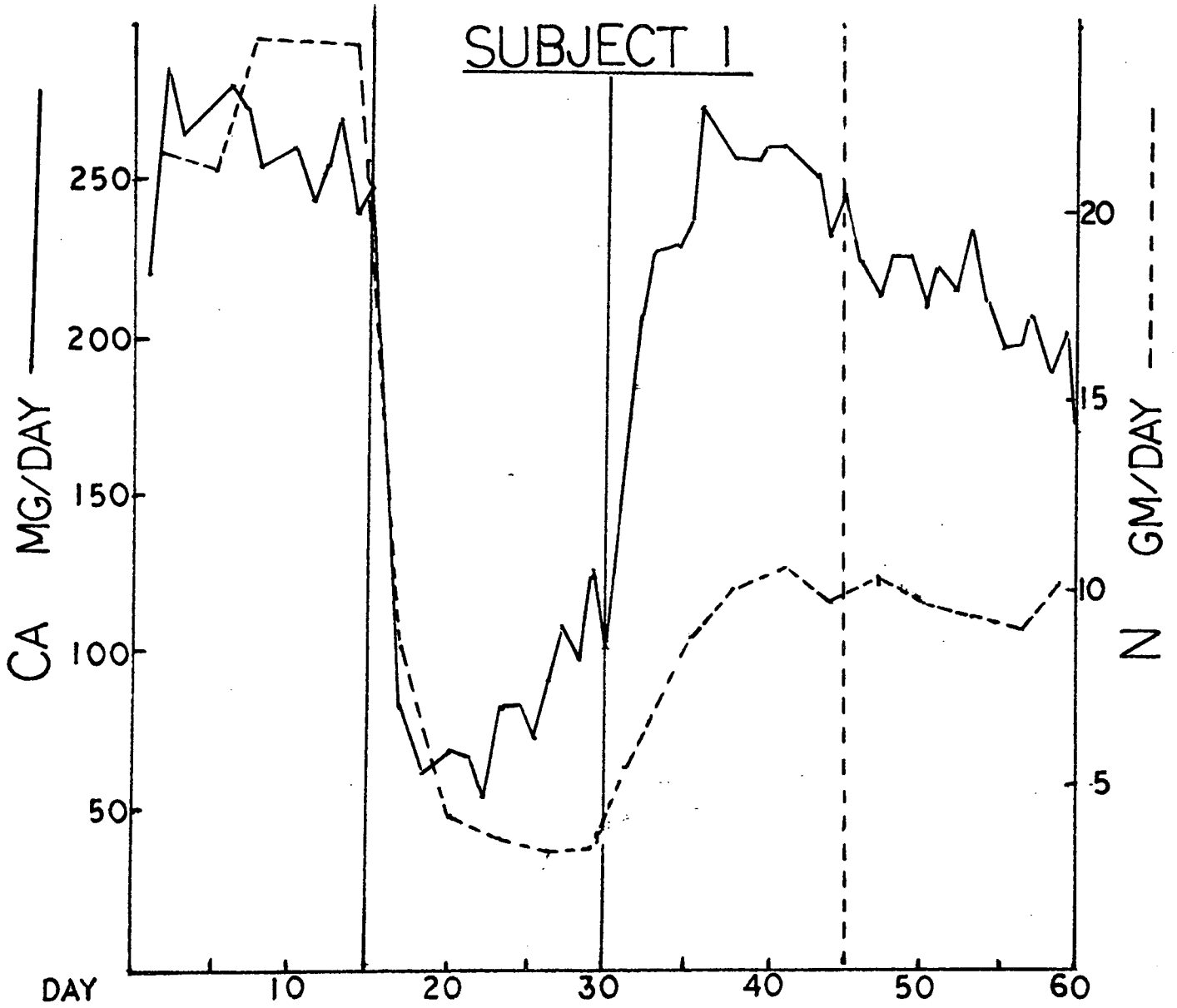
[g] Mean and S.D. of individual average of subject 3,4,5 and 6

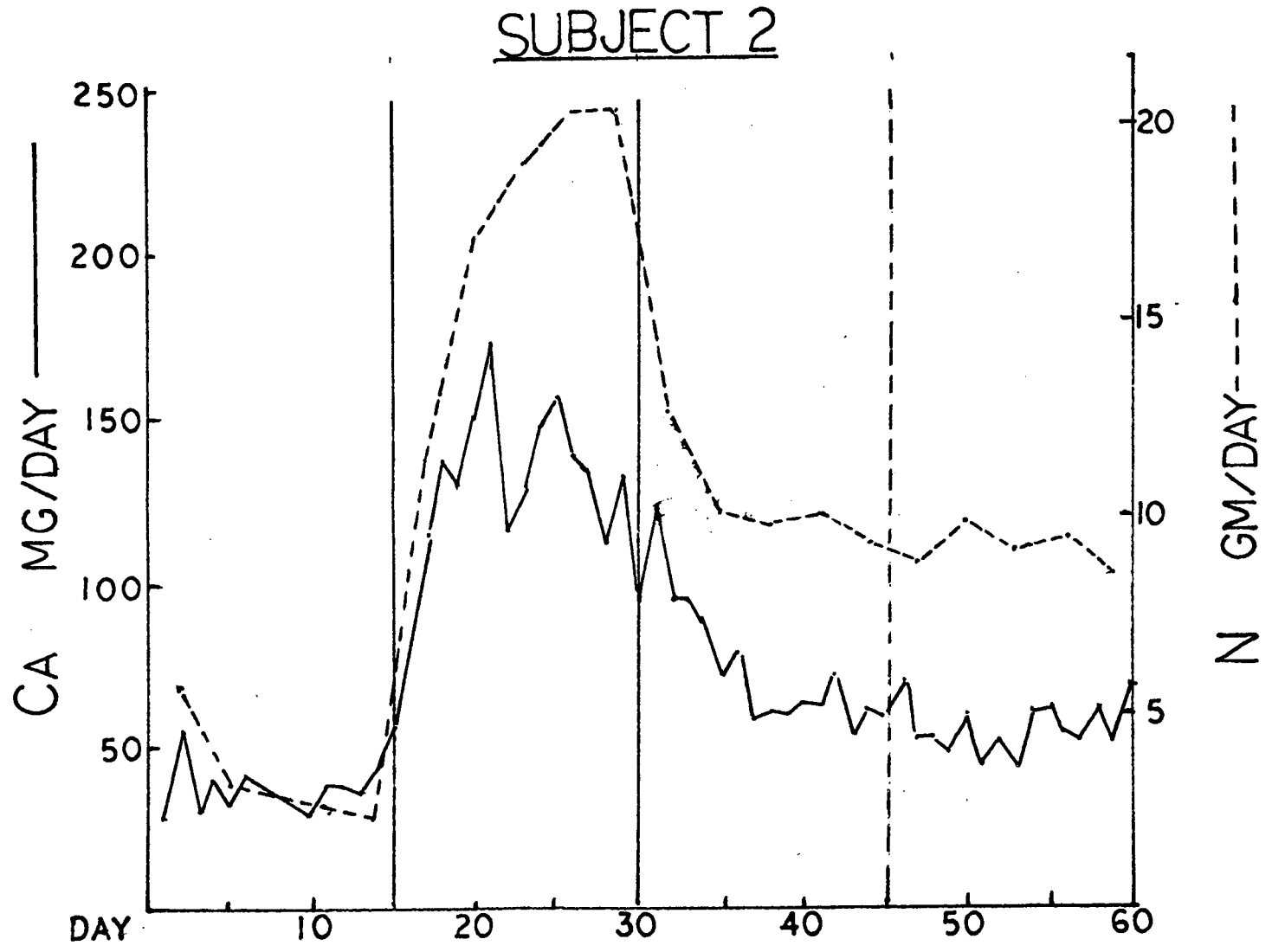
[h] " " 3,5 and 6 (subject 4 was not included because there was possible error on recording and gave high fecal calcium during period 4)

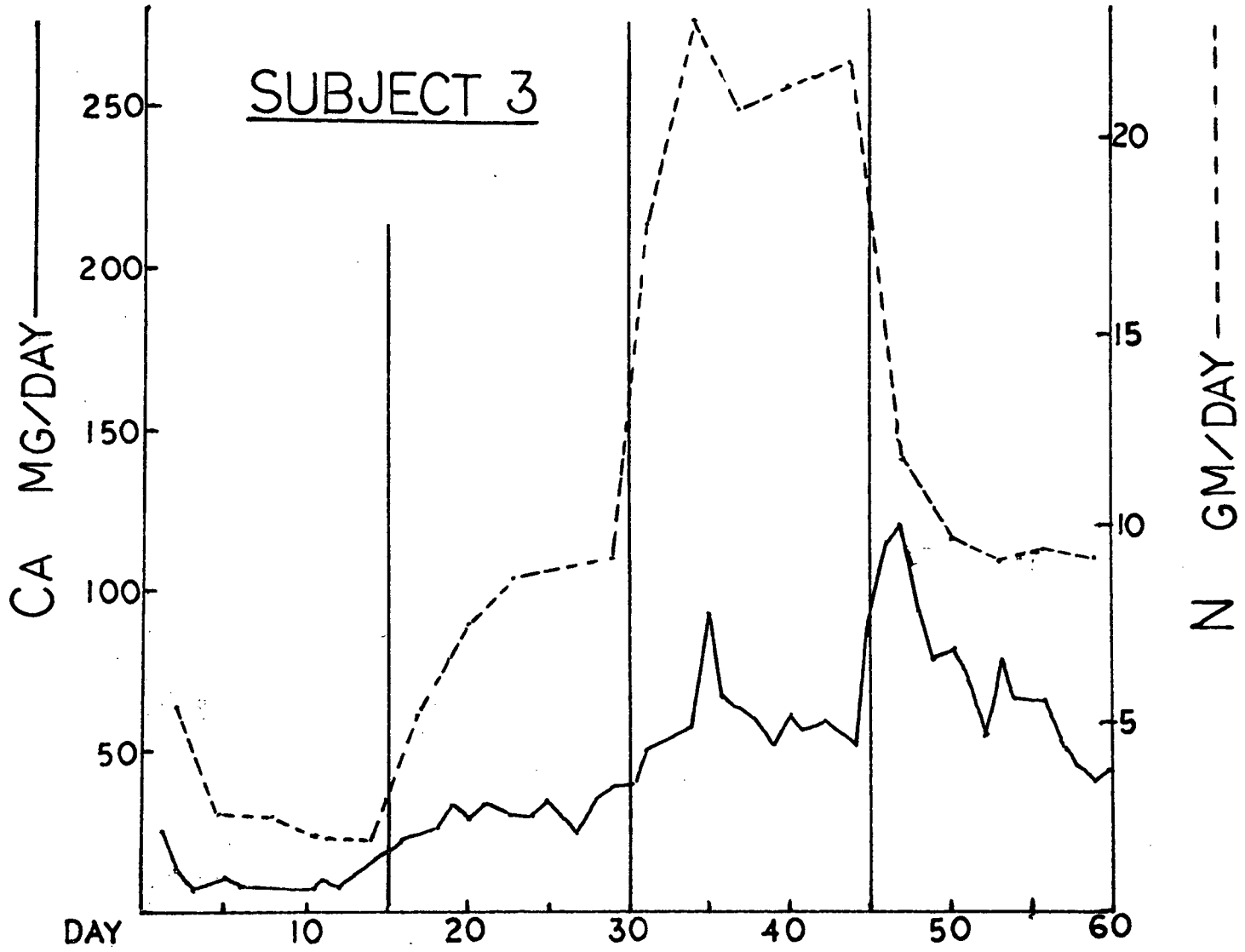
[i] Mean and S.D. of individual average of subjects 2,3,4,5, and 6

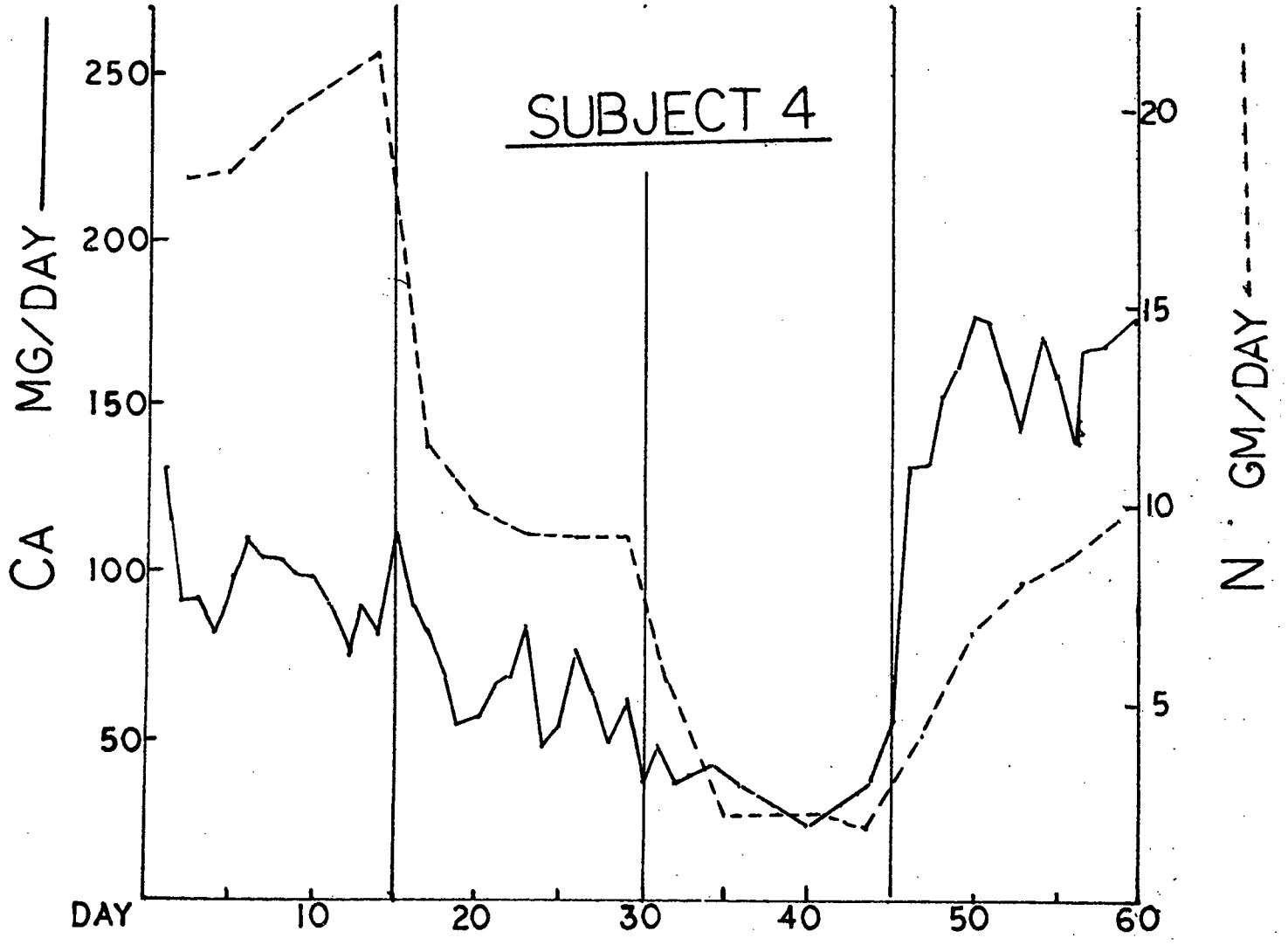
Figure 1a-f

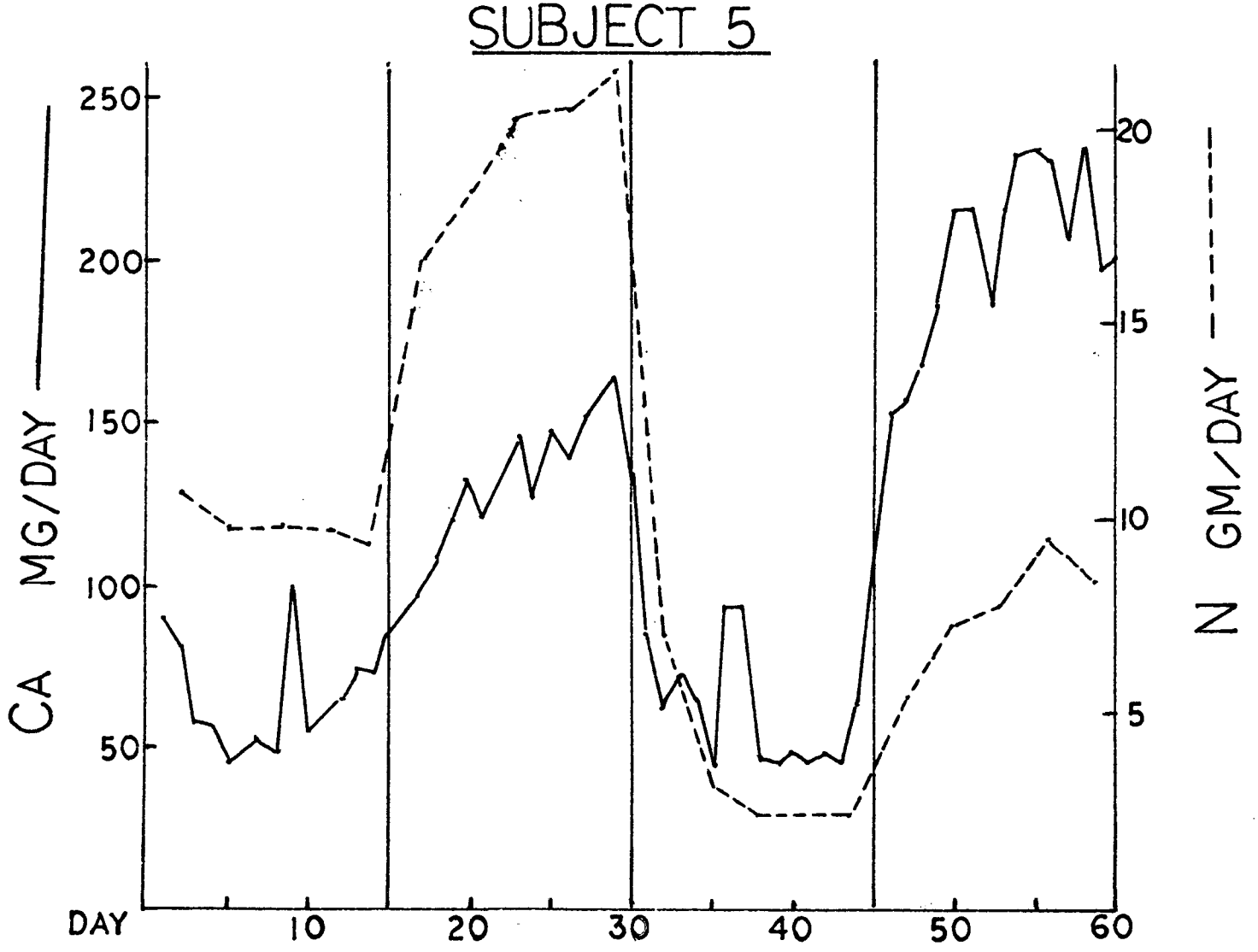
Urinary Excretion of Calcium and Nitrogen


SUBJECT 1
CA MG/DAY
N GM/DAY
250
200
150
100
50
20
15
10
5
DAY
10
20
30
40
50
60

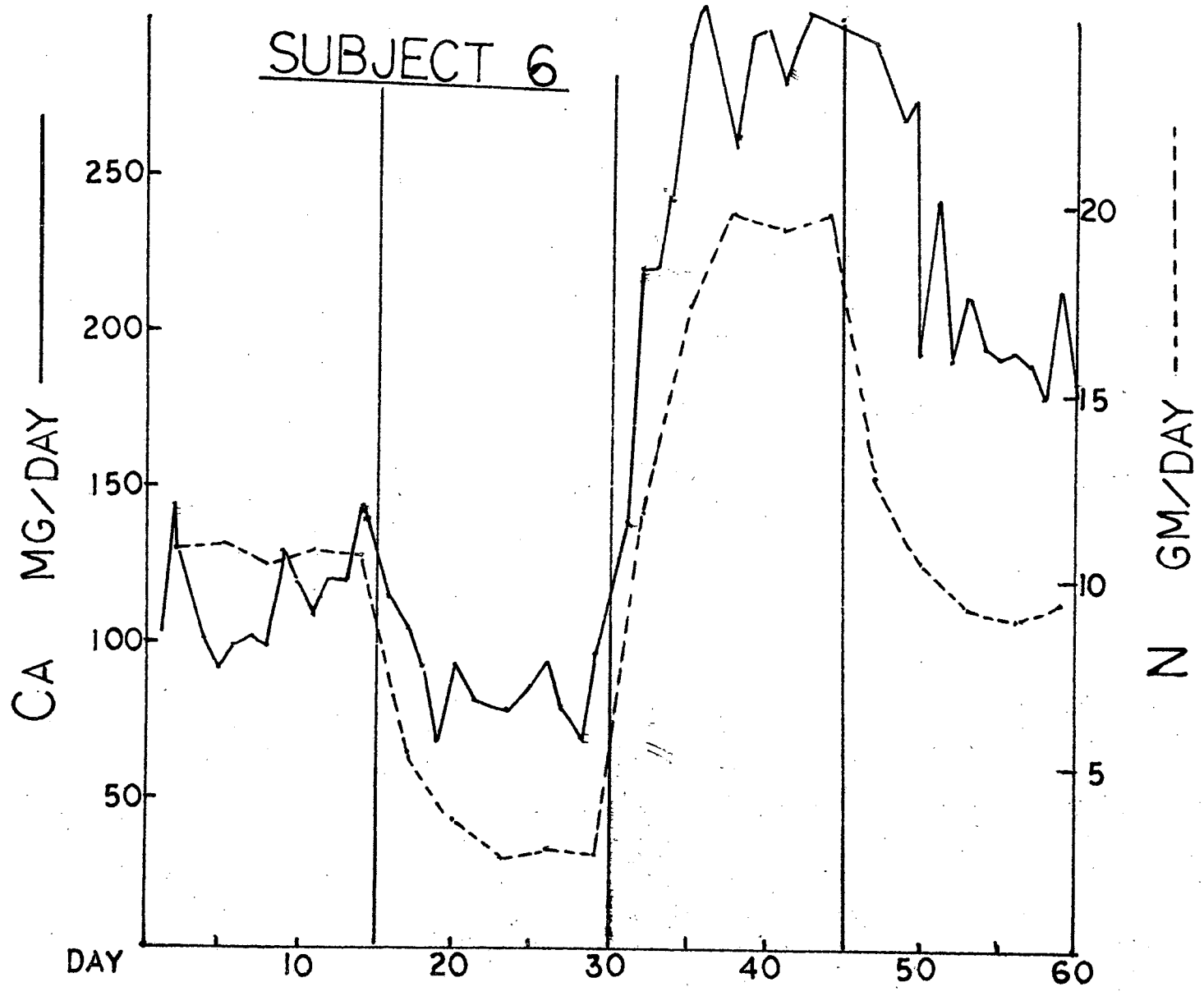

SUBJECT 2
CA MG/DAY
N GM/DAY
250
200
150
100
50
20
15
10
5
DAY
10
20
30
40
50
60


SUBJECT 3
CA MG/DAY
N GM/DAY
250
200
150
100
50
20
15
10
5
DAY
10
20
30
40
50
60


SUBJECT 4
CA MG/DAY
N GM/DAY
250
200
150
100
50
20
15
10
5
DAY
10
20
30
40
50
60


SUBJECT 5
CA MG/DAY
N GM/DAY
250
200
150
100
50
20
15
10
5
DAY
10
20
30
40
50
60

60-e


SUBJECT 6
CA MG/DAY
N GM/DAY
250
200
150
100
50
20
15
10
5
DAY
10
20
30
40
50
60

urinary calcium excretion was twice the amount of calcium ingested. The effect was even more pronounced in subject 6 who excreted 3.3 times the amount of calcium ingested. Although subject 3 did not excrete more calcium than he ingested during the high protein diet period, his excretion during this period was 6.5 times higher than during the protein-free diet period (Table 12).

The calcium supplement also increased the urinary calcium considerably. The additional intake of 900 mg of calcium daily with a medium-protein diet resulted in an average of 90 mg per day increase in daily urinary calcium excretion (Table 12).

In contrast to the urinary excretion, the sweat losses of calcium were not influenced by the dietary protein and calcium levels. The average daily loss was 15 mg (range 5-36 mg). The amount was about 11%, 15%, 25% and 9% of the amount in the urine during the high-protein, medium-protein, protein-free and medium-protein plus calcium supplement periods, respectively. The amount excreted in sweat for suject 3 was even greater than the amount in the urine during the protein-free diet period.

The study of sweat collected after strenous exercise revealed a large loss during this short time period, an average of 25 mg calcium loss in a 40-minute interval (Table 13). The amount of calcium lost through sweat and the calcium concentration (i.e. amount of calcium

Table 13 Calcium Losses during Strenous Exercise[a]

Subject	Diet	Period Order	Weight Loss	Calcium Loss	Calcium Concentration in Sweat[b]
			gm/interval	mg/interval	mg/kg
1	0.9N	2	220	19	86
	12N	3	260	38	146
	12N[c]	4	310	32	103
	24N	1	280	34	121
2	0.9N	1	340	23	68
	12N	3	--	--	--
	12N[c]	4	240	27	113
	24N	2	310	19	61
3	0.9N	1	290	13	45
	12N	2	230	27	117
	24N	3	270	29	107
	12N+Ca	4	280	13	46
4	0.9N	3	270	18	67
	12N	2	200	11	55
	24N	1	--	--	--
	12N+Ca	4	180	15	83
5	0.9N	3	340	29	85
	12N	1	320	42	131
	24N	2	320	11	34
	12N+Ca	4	350	17	49
6	0.9N	2	270	15	56
	12N	1	250	42	168
	24N	3	290	24	83
	12N+Ca	4	280	27	96

[a]Study at end of each period

[b]Calcium loss divided by body weight loss

[c]Diet was not changed between periods 3 and 4

divided by body weight loss) were not related to the protein and calcium intake or urniary calcium excretion.

Fecal calcium decreased significantly as protein intake increased from 0.9 to 12 gm nitrogen per day, but there was no further decrease as protein intake increased to 24 gm nitrogen per day. The decrease in fecal calcium did not parallel the increase in urinary calcium (Table 12).

The calcium balance calculated from the average value for each period is shown in Table 12. All but subject 1 showed the same trend, that is, they lost the least amount of calcium during the medium-protein diet period. There was no substantial difference in calcium balance between protein-free and high-protein diets periods for subjects 2,3 and 4. The negative balance during high-protein diet period was almost twice that of protein-free diet period for subject 6. Subject 1, however, had the least negative balance during the protein-free diet period. This was a result of the sharp increase in urinary calcium during the periods of medium- and high-protein intake. High calcium supplements reversed the negative balances to positive balances, but subjects 3, 5 and 6 had an average of only 32 mg per day positive balance after a long period of negative balance (an average of 150 mg calcium loss per day for 45 days).

The level of serum total calcium and alkaline phosphatase was quite steady throughout the whole experiment

regardless of protein and calcium intake (Table 14). Individual variation was smaller for serum calcium than that for alkaline phosphatase. No correlation was found between either calcium or alkaline phosphatase and the individual excretion of calcium in urine, feces or sweat.

There was marked individual variation in urinary hydroxyproline excretion (Table 15). On the protein-free diet the daily amount excreted was 21.0 to 80.2 mg per day (average 46.2 mg). The medium- and high-protein diets increased the daily excretion to 39.8 to 111.8 mg (average 66.5 mg) and 63.3 to 142.7 mg (average 98.3 mg), respectively. However, the medium-protein diet provided 0.73 gm and the high-protein diet provided 1.36 gm hydroxyproline daily. Calcium supplementation decreased the urinary hydroxyproline significantly for subjects 3, 4, 5 and 6 when compared with the period which had the same diet without the calcium supplement. However, the same kind of decrease was also found in subjects 1 and 2 during period 4 although these subjects did not receive calcium supplements.

Creatinine clearance rate is a convenient and fairly good method to evaluate the glomerular filtration rate. Creatinine clearance rates calculated from daily creatinine excretion and plasma creatinine level are shown in Table 16. There was a wide variation in creatinine clearance rate between individuals. The average creatinine clearance

Table 14 Some Fasting Blood Constituents[a]

Subject	Diet	Order	Calcium		Phosphorus		Mg.	Alk. P-ase	Creatinine	Urea N.	Glu.
			(mg %					)(IU %)(	mg%		)
1	---[b]		9.6		4.1		2.0	34	1.0	15.5	94
	0.9N	2	9.7	9.9	4.2	3.9	1.9	36 37	1.1	5.0	82
	12N[c]	3	9.3	10.1	4.3	4.0	2.2	34 36	1.2	13.5	90
	12N[c]	4	9.4	10.2	4.2	4.6	2.1	37 43	1.2	13.0	85
	24N	1	9.7	9.8	4.2	3.8	2.2	40 36	1.3	19.5	84
2	---		9.4		5.1		2.2	25	1.0	15.0	102
	0.9N	1	9.1	9.0	4.9	4.9	2.1	27 25	1.1	4.0	98
	12N[c]	3	9.7	9.1	5.3	4.9	2.1	27 29	1.1	10.5	92
	12N[c]	4	9.4	9.0	5.0	5.7	2.2	28 32	1.0	12.0	86
	24N	2	9.9	9.4	5.4	5.0	2.3	22 25	1.1	17.5	96
3	---		9.6		4.6		2.0	20	0.9	19.0	104
	0.9N	1	9.6	9.3	4.3	3.8	2.1	28 28	1.4	4.5	96
	12N	2	9.6	9.9	4.1	4.2	2.2	22 24	1.4	14.5	95
	24N	3	9.2	9.7	4.2	3.9	2.1	23 26	1.3	24.5	92
	12N+Ca	4	9.6	9.0	4.3	4.3	2.3	28 30	1.2	15.5	94
4	---		9.0		4.3		1.9	14	1.1	19.0	90
	0.9N	3	9.0	9.6	4.6	4.3	1.8	15 17	1.3	5.0	86
	12N	2	9.8	9.7	4.8	4.7	2.0	15 15	1.4	13.0	94
	24N	1	9.7	9.5	4.4	4.7	1.9	16 14	1.4	19.5	84
	12N+Ca	4	9.2	9.5	4.9	4.3	1.8	16 20	1.4	12.5	90
5	---		9.3		3.9		1.9	31	1.0	16.0	100
	0.9N	3	9.8	9.5	4.2	4.1	1.9	43 47	1.2	4.5	78
	12N	1	9.8	9.4	4.2	4.0	2.1	-- 33	1.3	11.5	88
	24N	2	9.7	9.7	4.0	4.0	2.2	34 34	1.1	19.0	98
	12+Ca	4	9.0	9.5	3.9	3.9	1.9	39 41	1.2	11.0	86
6	---		9.1		5.5		1.8	33	1.1	15.0	104
	0.9N	2	9.5	9.5	5.3	4.3	1.9	37 36	1.4	5.0	92
	12N	1	9.1	9.2	4.7	5.2	1.9	35 33	1.3	12.5	84
	24N	3	8.8	9.2	5.2	4.5	1.8	27 30	1.3	16.0	82
	12N+Ca	4	9.1	9.2	5.1	4.9	2.0	36 37	1.3	12.0	90

[a] Results for magnesium(Mg.), creatinine, urea N, glucose(Glu.) from fasting blood samples at the end of each period, results for calcium, phosphorus and alkaline phosphatase(Alk. P-ase) from samples at day 8 and at the end of each period.

[b] Fasting blood sample of first day, diets before admission was not controlled.

[c] Diet was not changed between periods 3 and 4.

Table 15 Urinary Hydroxyproline Excretion[a]

Subject	Diet	Period Order	Day of Period 1-3	4-6	6-9	10-12	12-15	Mean
			mg/day					mean±S.D.
1	0.9N	2	--	80.4	76.7	81.2	82.6	80.2 2.5
	12N[b]	3	107.1	110.1	111.4	120.6	108.6	111.8 5.3
	12N[b]	4	103.6	90.5	100.9	113.7	95.7	100.9 8.6
	24N	1	153.0	145.5	138.0	142.2	13218	142.3 7.6
2	0.9N	1	42.9	41.3	42.0	43.3	41.3	42.2 0.9
	12N[b]	3	73.4	73.4	65.5	68.8	69.5	70.1 3.3
	12N[b]	4	60.3	58.1	62.9	70.8	62.7	63.0 4.8
	24N	2	88.8	90.2	96.4	108.2	90.0	94.9 8.0
3	0.9N	1	18.3	17.4	23.6	22.3	23.6	21.0 3.0
	12N	2	48.5	45.9	48.9	43.3	47.3	46.8 2.3
	24N	3	85.6	96.4	78.7	73.4	80.0	82.8 8.7
	12N+Ca	4	38.0	48.5	42.0	44.6	50.7	44.8 5.1
4	0.9N	3	27.5	22.7	25.6	23.6	26.2	25.1 1.9
	12N	2	43.3	35.4	37.1	41.6	41.6	39.8 3.4
	24N	1	59.0	64.9	64.9	61.0	66.9	63.3 3.2
	12N+Ca	4	35.0	36.7	35.1	35.4	43.7	37.2 3.7
5	0.9N	3	56.0	57.7	45.2	45.2	47.6*	50.3 6.1
	12N	1	55.1	53.5	59.4	64.9	61.2	58.7 4.7
	24N	2	98.3	90.5	95.7	92.4	98.3	95.0 3.5
	12N+Ca	4	50.7	59.0	55.1	59.0	59.0	56.6 3.7
6.	0.9N	2	65.5	54.1	53.1	61.0	58.1	58.4 5.1
	12N	1	62.9	68.8	79.4	77.0	69.9*	71.6 6.6
	24N	3	120.5	111.8	106.6	103.2	114.1*	111.3 6.8
	12N+Ca	4	66.5	61.2	58.1	59.0	64.9	61.9 3.7
Mean[c]	0.9N							46.2 22.0
	12N							66.5 25.5[d]
	24N							54.2 14.0[d]
	12N+Ca							98.3 26.8[d]
								50.1 11.2[d]

[a] From 3-day pooled urine samples except 2 samples which were 2-day pooled urine(marked with *). Hydroxyproline content in diet: 0.9N diet, free of hydroxyproline, 12N diet, 0.73 gm/day and 24N diet, 1.36 gm/day

[b] Diet was not changed between period 3 & 4 for subjects 1 & 2

[c] Mean and S.D. of individual average of 6 subjects

[d] " " subjects 3,4,5 & 6.

Table 16 Urinary Creatinine, Creatinine Clearance Rate and Calcium Excreation per Unit Volume of Glomerular Filtrate

Subject	Diet	Order	Creatinine Excretion (U_{cr}) gm/day mean+S.D.		U_{cr} mg/Kg/day	C.C.R. (C_{cr})[a] ml/min	C.C.R. ml/min/1.73 m^2	C.E.G.F. (Ca_E)[b] mg/100 ml G.F.
1	0.9N	2	2.01	0.06	24.6	126.9	112.6	0.047
	12N	3	2.15	0.05	26.5	124.4	110.4	0.141
	12N	4	2.16	0.06	26.6	125.0	110.9	0.125
	24N	1	2.48	0.08	30.2	132.5	117.6	0.135
2	0.9N	1	1.75	0.26	21.9	110.5	102.8	0.024
	12N	3	2.10	0.05	26.3	132.6	123.3	0.034
	12N	4	1.96	0.36	24.7	136.1	126.6	0.028
	24N	2	2.21	0.16	27.9	139.5	129.8	0.067
3	0.9N	1	1.25	0.04	21.7	62.0	69.2	0.008
	12N	2	1.47	0.04	25.5	72.9	81.4	0.031
	24N	3	1.83	0.12	31.7	97.8	109.2	0.046
	12N+Ca	4	1.53	0.02	26.5	88.5	98.8	0.049
4	0.9N	3	1.76	0.10	26.7	94.0	96.8	0.024
	12N	2	1.92	0.04	29.1	95.2	98.0	0.043
	24N	1	2.01	0.08	30.7	99.7	102.7	0.067
	12N+Ca	4	1.84	0.02	28.0	91.3	94.0	0.127
5	0.9N	3	1.86	0.03	28.2	107.6	108.9	0.037
	12N	1	1.97	0.04	30.1	105.2	106.4	0.040
	24N	2	2.27	0.11	34.5	143.3	145.0	0.067
	12N+Ca	4	1.92	0.04	29.0	111.1	112.4	0.119
6	0.9N	2	1.95	0.05	27.6	96.7	94.5	0.060
	12N	1	2.16	0.16	30.3	115.4	112.8	0.070
	24N	3	2.42	0.12	34.2	128.3	125.4	0.152
	12N	4	2.27	0.21	31.9	121.3	118.6	0.116
Mean[c]	0.9N				mean±S.D. 25.1 2.8	mean±S.D. 97.5 15.5	mean ± S.D. 0.033	0.019
	12N				28.0 2.1	105.4 14.4	0.060	0.042
					(28.8 2.2	99.7 5.9	0.046	0.017)[d]
	24N				31.5 2.5	121.6 15.2	0.089	0.043
	12N+Ca				(28.9 2.3	106.0 11.5	0.103	0.036)[d]

[a] Creatinine Clearance Rate (C.C.R.), calculated by the equation:

$$C_{cr} = \frac{U_{cr}}{P_{cr}} \times \frac{1}{1440}$$

, where P_{cr} serum creatinine level

(Table 16 continued)

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[b]Calcium Excretion per 100ml Glomerular Filtrate (C.E.G.F.), calculated by the equation:

$$Ca_E = \frac{V_u}{U_{cr}} \times P_{cr}$$, where V_u daily urinary calcium excretion

[c]Mean and standard deviation of the average of 6 subjects or otherwise specified.

[d]Mean and standard deviation of the average of subjects 3,4, 5 and 6.

rate increased from an average of 97.5 ml/min./1.73 m^2 body surface area with protein-free diet to 105.4 and 121.6 ml during medium- and high-protein diet, respectively.

An analysis of the dynamics of calcium excretion must take into account interindividual variation and changes in glomerular filtration rate induced by variable protein. This can be done by relating calcium output to a fixed volume of glomerular filtrate (G.F.). Calculated amount of calcium excreted per 100 ml of G.F. are shown in Table 16. There was also remarkable individual variation. In all but one period of subject 1, calcium excretion per 100 ml G.F. increased as the protein intake increased. The additional intake of 900 mg calcium daily with a medium-protein diet resulted in an average increase of more than twice the amount excreted compared with the period of same diet without calcium supplements.

^{47}Calcium studies

The results of the ^{47}calcium studies are shown in Table 17 and 18. The calcualted calcium fractional absorption rates, endogenous fecal calcium, digestive juice calcium, calcium miscible pool, rate of calcium entering and leaving bone are shown in Table 19 and 20. Table 20 shows the results obtained by using two different methods of calculation for the same study. Difference can be noted depending upon the approach used. Good agreement

Table 17 Results of Intravenous ^{47}Calcium Study

Subject	Diet	Calcium Ingested (V_i)	Urinary Calcium[a] (V_u)	^{47}Calcium in Urine[b] (R_u)	^{47}Calcium in Feces[c] (R_F)	^{47}Calcium Ratio (R_F/R_u)
		(mg/day)		(% dose)		
1	0.9N	101	83	4.0	6.3	1.58
2	24N	87	137[d]	9.0[d] 10.0	8.2[d]	0.91[d]
3	12N	105	30	4.0	13.3	3.33
4	12N	105	66	7.1	8.0	1.13
5	24N	88	142	11.4	4.8	0.42
6	0.9N	101	81	6.1	9.1	1.49

[a]Average urinary calcium of day 1-6 after injection

[b]Recovery of radioactivity in urine of day 1-6 after injection

[c]Recovery of radioactivity in feces of day 2-7 after injection

[d]No feces on day 7, urine of day 1-5 and feces of day 2-6 used for calculation

Table 18 Results of Oral ^{47}Calcium Study

Subject	Diet	V_i[a]	R_F[b]	R_u[c]	$\frac{R_u}{100-R_F} \times 100$	S.A.$_i$	S.A.$_u$[d]	S.A.$_F$[e]	$\frac{S.A._i}{S.A._F}$
		mg/day	(% Dose)			(x 10^{-5} cpm/gm Ca.)			
1	12N	107	15.2	2.8	11.9	22.55	0.940	2.805	8.0
2	12N	107	29.7[f]	3.1[f]	4.4[f]	22.55	1.167	5.824	3.9
3	24N	85	40.6	3.5	5.9	28.39	1.494	9.327	3.0
4	0.9N	98	35.5	1.7	2.6	24.26	1.372	4.836	5.0
5	0.9N	101	20.5	2.7	3.4	23.89	1.189	4.175	5.7
6	24N	88	29.4	10.9	15.4	27.42	1.014	6.679	4.1

[a] Notation see Table 10

[b] Recovery of radioactivity in feces of day 2-11 after ^{47}calcium ingestion

[c] " " urine of day 1-10 after ^{47}calcium ingestion

[d] Average specific activity of urine of day 5-7 after ^{47}calcium ingestion

[e] Average specific activity of feces of day 6-8 after ^{47}calcium ingestion

[f] No feces on day 11, urine of day 1-10 and feces of day 2-11 used for calculation

Table 19 Kinetics Data of Calcium Metabolism [a]

Subject	Diet	V_{ef}	α	V_d	m	P	V_T	V_{o+}	V_{o-}	P	V_T	V_{o+}	V_{o-}
		mg/day	%	mg/day	day^{-1}	mg	(mg/day		)mg/m^2		(mg/day/m^2)		
1	0.9N	131	89	1190	0.241	6506	1568	1334	1478	3318	804	684	758
2	24N	125	75	500	0.199	5010	995	733	932	2689	535	394	501
3	12N	100	41	169	0.189	2724	515	372	472	1757	332	240	305
4	12N	75	52	156	0.107	4815	515	370	460	2866	307	220	274
5	24N	60	81	316	0.211	4531	956	749	887	2650	558	437	518
6	0.9N	121	38[b] 57[b]	195[b] 281[b]	0.292	3842	1122	912	1084	2171	634	515	612

[a] Calculated from intravenous ^{47}calcium study, notation see page 51 Table 10

[b] Calculated not based on fecal calcium of whole period, based on fecal calcium of day 7-14 of period 2

Table 20 Endogenous Fecal Calcium, Digestive Juice Calcium and Fractional Absorption Rate[a]

Subject	Diet	V_d		α		V_{ef}	
		mg/day		%		mg/day	
		I[b]	II[c]	I[d]	II[e]	I[f]	II[g]
1	12N	1134	2083	89	94	125	125
2	12N	381	474	68	73	122	128
3	24N	206	180	55	51	93	88
4	0.9N	557	364	70	58	167	153
5	0.9N	668	453	81	74	127	118
6	24N	325	272	71	67	94	90

[a]Calculated from oral ^{47}calcium study

[b]Calculated from equation 7

[c]Calculated from equation 3 using values of α(II) and V_{ef}(II)

[d]Calculated from equation $\alpha=(V_i+V_d-V_F)/(V_i+V_d)$, using values of V_d(I)

[e]Calculated from equation 2 using values of V_{ef}(II)

[f]Calculated from equation $V_{ef}= V_d x(1 -\alpha/100)$ using values of α(I) and V_d(I)

[g]Calculated from equation 8

was noted between results for calculated endogenous fecal calcium and for fractional absorption rate. However, the results for digestive juice calcium varied considerably.

The average fractional absorption rate was about 70% during the low calcium intake periods. It ranged from 41 to 94 %. The endogenous fecal calcium was between 60 and 167 mg per day. Table 21 shows the comparison of some calculated results from the two studies. Although different methods were employed in the two studies, the results demonstrate the effect of dietary protein on the calcium absorption in the gut. With the exception of subject 4, all showed an increase in calcium absorption and a decrease in endogenous fecal calcium during the higher protein intake. There was no such consistent correlation between protein intake and digestive juice calcium.

Semilogarithmic plots of the specific activity of ^{47}calcium in the urine against time for the intravenous ^{47}calcium study are shown in Figure 2a-c. The slope of the regression line gives the turnover of calcium pool which is expressed as a fraction of the pool replaced per day. Daily turnover of the pool ranged between 0.109 to 0.292. The parameters of calcium metabolism calculated from these results and from the calcium balance are shown in Table 19. The rate of calcium entering bone ranged from 372 mg to 1334 mg per day; and the rate of calcium

Table 21 Comparison of Fractional Absorption Rate, Endogenous Fecal Calcium and Digestive Juice Calcium between Two Periods

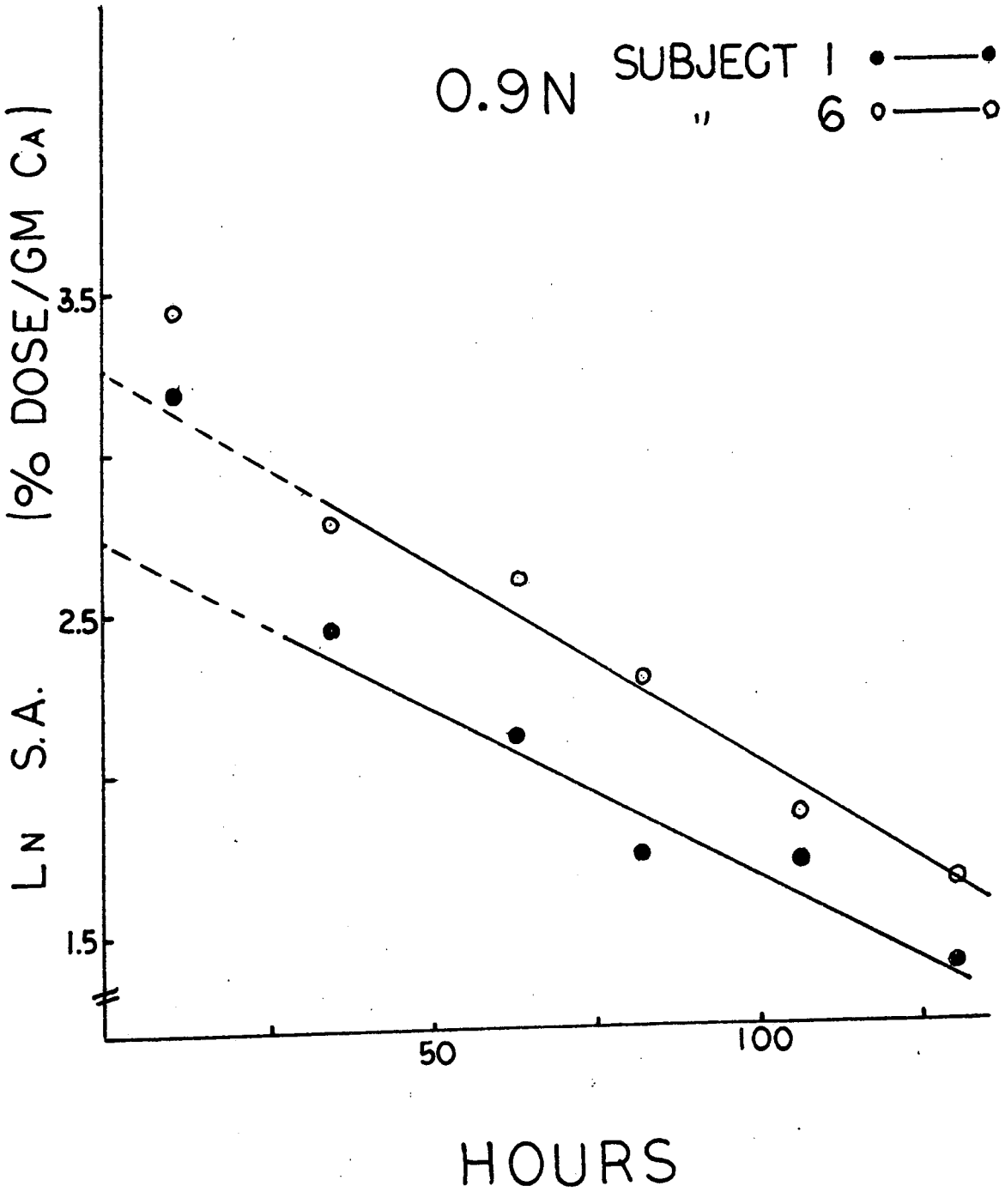
Subject	Diet		α		V_{ef}		V_d	
	II	III	II	III	II	III	II	III
A. Group that protein intake increased								
1	0.9N	12N	89	92(94, 89)[a]	131	125(125, 125)	1190	1613(2083, 1134)
3	12N	24N	41	53(51, 55)	100	91(88, 93)	169	193(180, 206)
6	0.9N	24N	57	69(67, 71)	121	92(90, 94)	281	299(272, 325)
B. Group that protein intake decreased								
2	24N	12N	75	71(73, 68)	125	125(128, 122)	500	428(474, 381)
5	24N	0.9N	81	78(74, 81)	60	123(118, 127)	316	561(453, 668)
4[b]	12N	0.9N	52	64(58, 70)	75	160(153, 167)	156	461(364, 557)

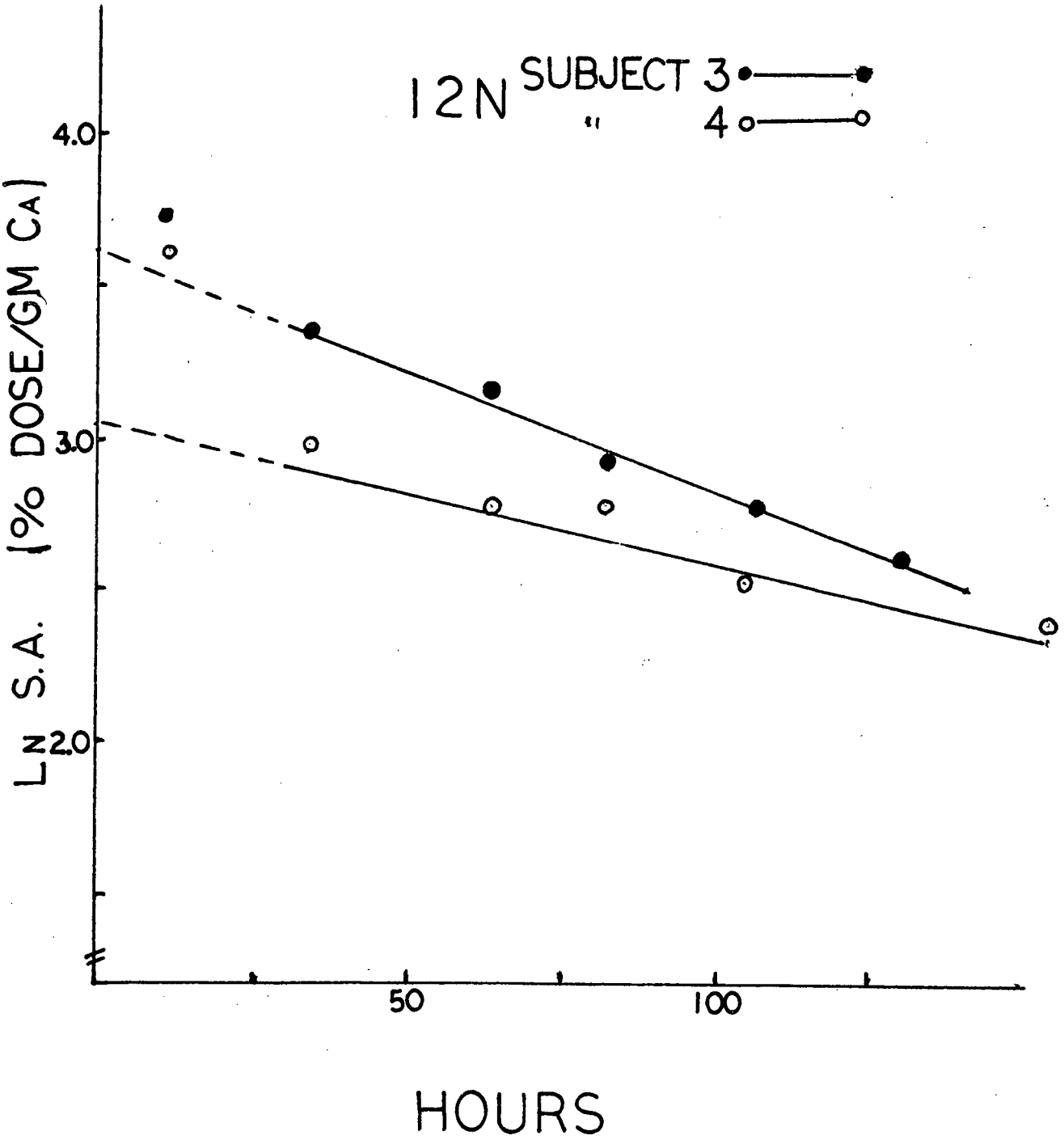
[a] Two methods used for calculation of these parameters(see Table 20), the value given before the parenthesis is the average of these two values

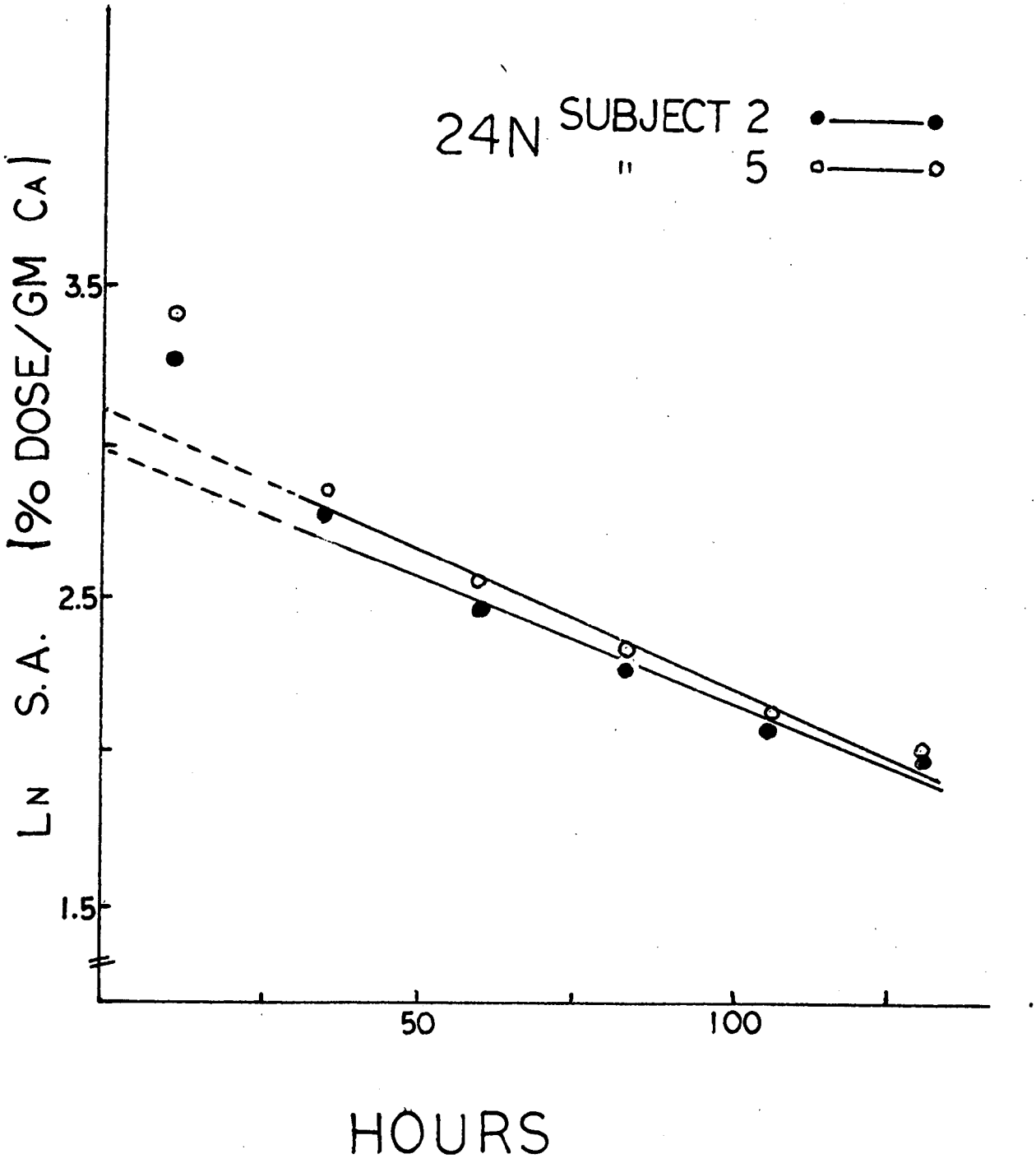
[b] The results of subject 4 during the period 2(II) by i.v. ^{47}calcium study were not satisfactory

Figure 2a-c

Semilogarithmic Plots of ^{47}Calcium Specific Activity Verus Time for Intravenous ^{47}Calcium study


0.9N
SUBJECT 1
" 6
LN S.A. (% DOSE/GM CA)
3.5
2.5
1.5
50
100
HOURS


12N SUBJECT 3
" 4
4.0
3.0
2.0
LN S.A. (% DOSE/GM CA)
50
100
HOURS


24N SUBJECT 2
" 5
LN S.A. (% DOSE/GM CA)
3.5
2.5
1.5
50
100
HOURS

leaving bone, from 515 to 1568 mg per day. Good correlation was found between V_{o-} or V_{o+} and the alkaline phosphatase or urinary hydroxyproline excretion of protein free period as shown Table 22.

The miscible calcium pool size calculated for this study varied from 2724 to 6506 mg. This pool size was shown to be correlated with body size. The correlation was slightly better with body surface ($r=0.84$) than with body weight ($r=0.76$).

Because of the wide variation in body size, the specific activity was corrected to body surface according to the method of Heaney et al. (192). The corrected specific activity plotted against time are given in Figure 3. Heaney et al. (192) combined the results of radiocalcium studies from various laboratories and calculated the mean and standard deviation of specific activity at various times after injection. The dashed-line area in Figure 3 represents the mean plus and minus one standard deviation of normal adults obtained from those calculations.

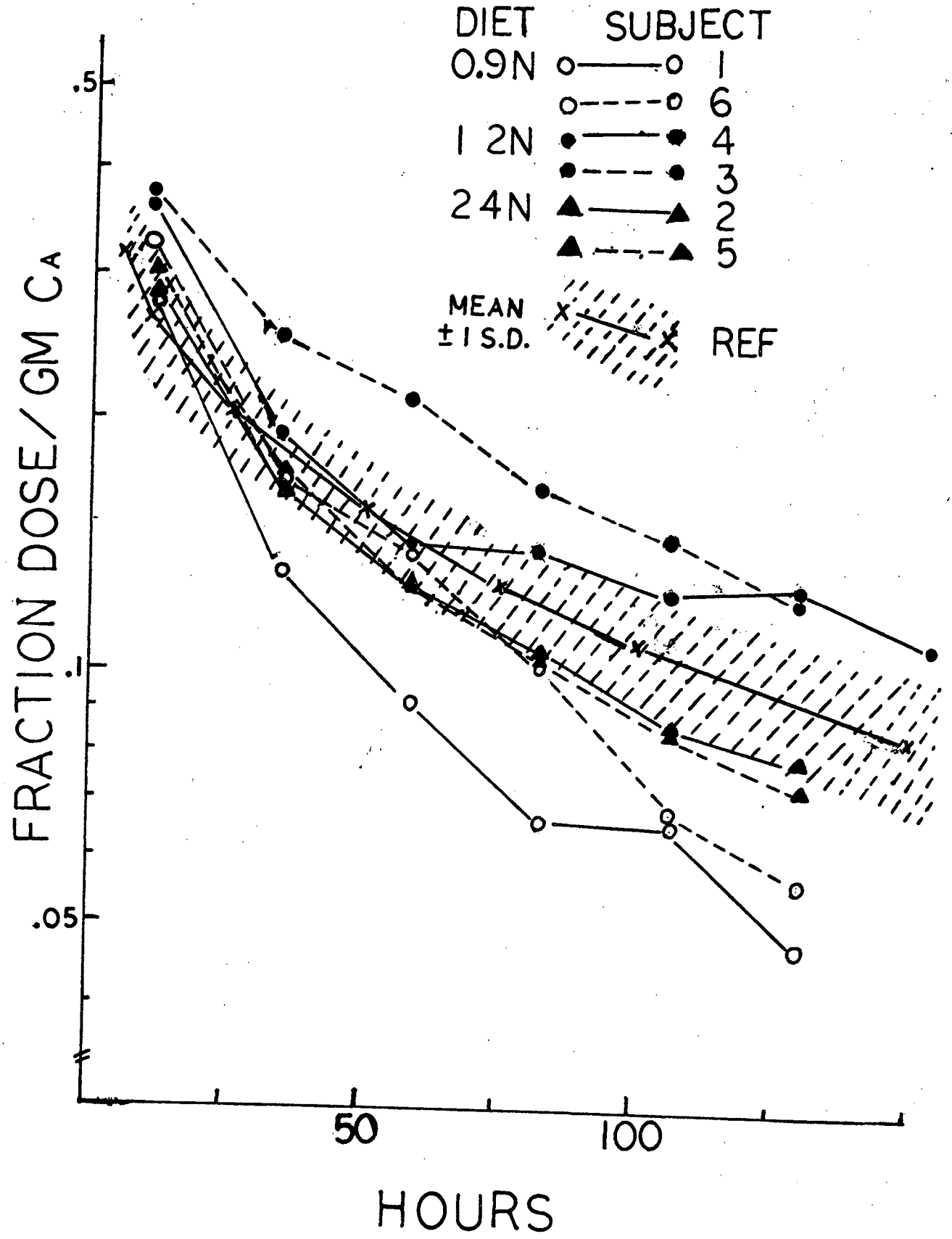
A comparison of the curves for out study with the reference standard of Heaney et al. (192, Figure 3) shows that the initial specific activity is higher than the mean but four out of six are within one standard deviation. Although the subjects started with specific activity similar to the reference, each subject had his

Table 22 Correlation of Rate of Calcium Entering or Leaving the Bone to Alkaline Phosphatase, Urinary Hydroxyproline Excretion

	Coefficient of Correlation	P
V_{o+} vs Serum alkaline Phosphatase	0.82	<0.05
V_{o-} vs Serum alkaline Phosphatase	0.82	<0.05
V_{o+} vs Urinary hydroxyproline of period 2	0.53	>0.1
V_{o-} vs Urinary hydroxyproline of period 2	0.57	>0.1
V_{o+} vs Urinary hydroxyproline of protein-free diet period	0.99	<0.001
V_{o-} vs Urinary hydroxyproline of protein-free diet period	0.99	<0.001

Figure 3

Corrected Semilogarithmic Plot of ^{47}Calcium Specific Activity Verus Time for Intravenous ^{47}Calcium Study


DIET
SUBJECT
0.9N
1
6
1 2N
4
3
2 4N
2
5
MEAN
± 1 S.D.
REF
.5
.1
.05
FRACTION DOSE/GM C_A
50
100
HOURS

own pattern of time course for the specific activity and none of them followed the reference standard.

The curves for subjects 3 and 4 are higher than the reference curves. The curve of subject 3 parallels the mean curve, but the slope of the curve for subject 4 starts to decrease on day 3. Subjects 2 and 5 have essentially the same curve but the slope of these curves is slightly steeper than the reference. The dilution of tracer proceeded more rapidly for subjects 1 and 6 than for subjects 2 and 5.

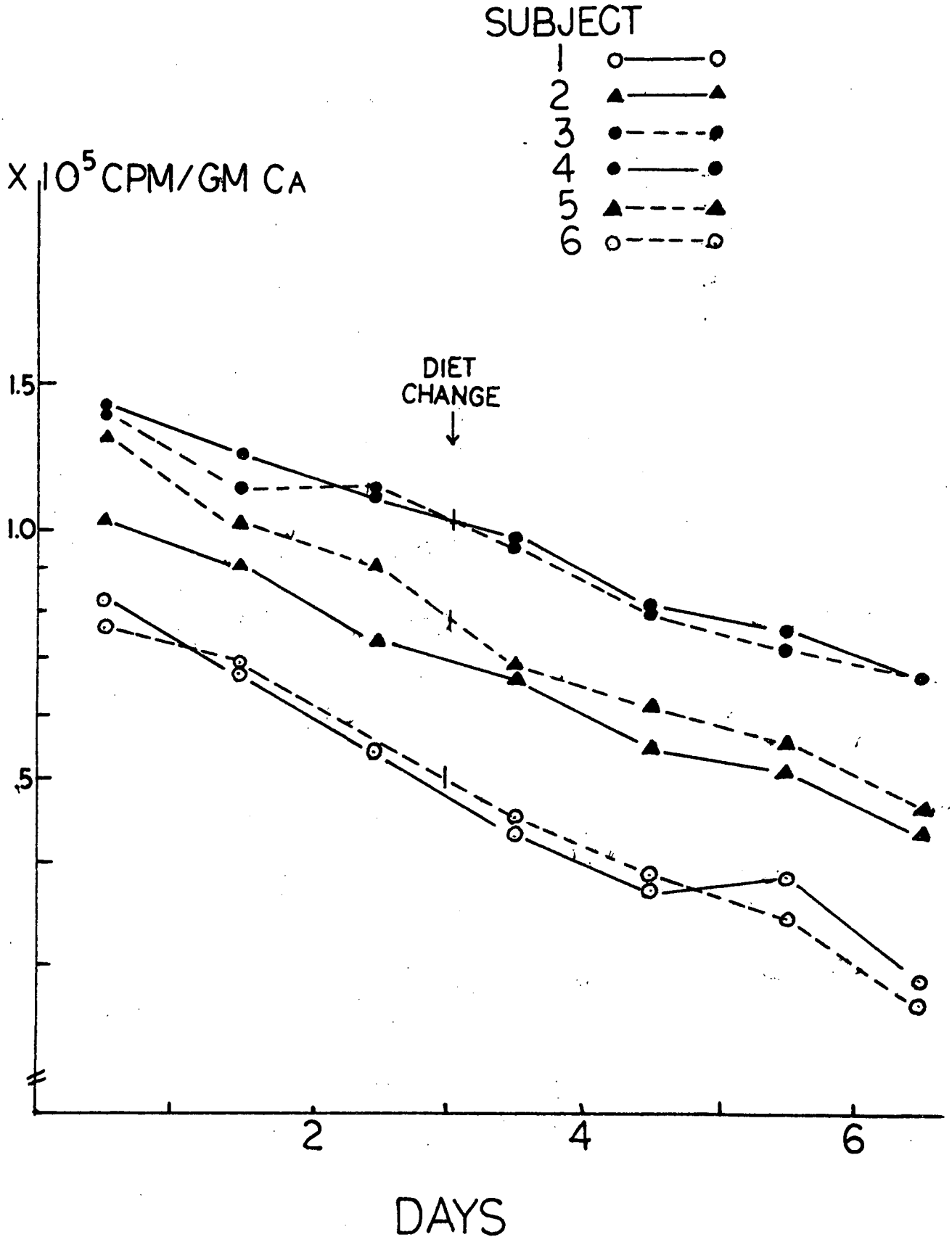
Similar plots for the oral ^{47}Calcium study after the cessation of oral radiocalcium feeding are shown in Figure 4. The diets of subjects 3, 4, 5, and 6 were changed to medium-protein diet with approximately 900 mg calcium supplementation on day 4. The slope of the regression line was calculated only for subjects 1 and 2 because their diet was not changed. The daily turnover of the calcium pool was 0.154 and 0.138 for subjects 1 and 2, respectively. The specific activity of ^{47}calcium in the urine was higher for subjects 3 and 4 and lower for subjects 1 and 6. In contrast to the curves in Figure 3, the slopes of the curves for different subjects did not vary greatly.

Nitrogen metabolism

The individual nitrogen intake, excretion and balance data are shown in Table 23. As expected, the urinary

Figure 4

Semilogarithmic Plot of ^{47}Calcium Specific Activity in Urine Verus Time for Oral ^{47}Calcium Study after Cessation of ^{47}Calcium Feeding


SUBJECT
1
2
3
4
5
6
X 10⁵ CPM/GM CA
DIET CHANGE
1.5
1.0
.5
2
4
6
DAYS

nitrogen was directly related to the protein intake. The calcium supplement for the medium-protein diet decreased the urinary nitrogen for three out of four subjects when compared to the period on the same diet without calcium supplementation. This difference was not significant probably because of the effect of the previous protein intake.

Fecal nitrogen, on the other hand, did not show a direct relationship to the protein intake. Although the average amount of fecal nitrogen increased as protein increased, the individual fecal nitrogen did not show such consistent relationship (Table 23). This result demonstrated the remarkably high digestibility of the meat protein used in this experiment and the ability of the gastrointestinal tract to tolerate easily at least 24 gm nitrogen meat protein. If we used the average fecal nitrogen during the first three diet periods, the digestibility calculated by the following equation (193):

$$\% \text{ digestibility} = \frac{\text{N Intake} - (\text{Fecal N}_{\text{test}} - \text{Fecal N}_{\text{protein-free}})}{\text{N Intake}}$$

would be 98% either during the medium- or high-protein diet period. The calcium supplement, however, increased the fecal nitrogen in all four subjects regardless of the order of dietary periods.

The apparent nitrogen balance defined as the difference between nitrogen intake and the excretion in urine,

Table 23 Nitrogen Intake, Excretion and Balance

Subject	Diet	Order	Nitrogen Intake[a]	Urinary Nitrogen[b]	Fecal Nitrogen[c]	Sweat Nitrogen[d]	Apparent Balance[e]
			gm/day				
				mean±S.D.			
1	0.9N	2	0.89	3.54 0.26	1.22	0.05[g]	-3.92
	$12N_f$	3	12.04	9.88 0.86	1.16	0.14	+0.86
	$12N^f$	4	12.04	9.67 0.47	1.26	0.17	+0.94
	24N	1	23.80	23.67 1.78	1.06	0.27	-1.20
2	0.9N	1	0.89	2.15 0.38	1.61	0.20	-3.07
	$12N_f$	3	12.04	9.84 0.35	1.58	0.16	+0.46
	$12N^f$	4	12.04	9.30 0.58	1.87	0.16[g]	+0.71
	24N	2	23.80	19.05 1.56	1.86	0.09[g]	+2.80
3	0.9N	1	0.84	2.23 0.25	1.14	0.13	-2.66
	12N	2	12.00	8.52 0.71	1.39	0.07[g]	+2.02
	24N	3	23.77	21.82 1.11	1.30	0.22	+0.43
	12N+Ca	4	11.96	9.27 0.26	1.76	0.24	+0.69
4	0.9N	3	0.84	2.15 0.16	0.88	0.08	-2.27
	12N	2	12.02	9.45 0.36	1.29	0.09[g]	+1.19
	24N	1	23.80	20.03 1.20	1.11	0.20	+2.46
	12N+Ca	4	12.00	8.29 1.28	1.42	0.16	+2.13
5	0.9N	3	0.89	2.56 0.41	1.15	0.06	-2.88
	12N	1	12.04	9.72 0.23	1.69	0.15	+0.48
	24N	2	23.80	20.22 1.30	2.18	0.07[g]	+1.33
	12N+Ca	4	12.04	7.82 0.47	1.78	0.11	+2.33
6	0.9N	2	0.89	2.76 0.30	1.11	0.05[g]	-3.03
	12N	1	12.07	10.70 0.25	1.55	0.13	-0.31
	24N	3	23.80	19.09 1.20	1.38	0.13	+3.20
	12N+Ca	4	12.04	9.45 0.76	1.82	0.13	+0.64
					mean±S.D.	mean±S.D.	
Mean[h]	0.9N			2.57 0.54	1.19 0.24	-2.97 0.50	
	12N			9.69 0.72	1.44 0.20	+0.78 0.79	
				(9.60 0.90	1.48 0.18	+0.85 0.99)[i]	
	24N			20.65 1.79	1.48 0.44	+1.50 1.67	
	12N+Ca			(8.71 0.78	1.67 0.17	+1.45 0.91)[i]	

[a] Based on analysis of composites plus individual calorie supplement

[b] Mean & S.D. of 3-day-pool samples fo day 4-15

[c] Average of fecal collection of day 4-15

[d] Sweat collected during day 8-14

(Table 23 Continued)

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[e]Blood loss and other unmeasureable losses were not included

[f]No dietary change between period 3 and 4

[g]Data for sweat nitrogen of period 2 was too low, probably a calculation error

[h]Mean and standard deviation of average of 6 subjects or otherwise specified

[i]Mean and standard deviation of average of subjects 3,4,5 and 6

feces, and sweat was positive for most subjects during the medium- and high-protein diet periods except for subjects 1 and 6. The negative balances for these two subjects might have been the result of a high protein intake before the experiment. The calcium supplement either increased or decreased the nitrogen retention as compared to the same dietary period without added calcium (Table 23).

Urinary creatinine excretion was also increased as the dietary protein intake increased. There was about 25% difference (ranged 15-45%) between the high-protein diet and the protein-free diet periods (Table 16). However, the fasting blood creatinine was essentially the same throughout the experiment.

Metabolism of other minerals:

The average intake, excretion and the apparent balance of sodium, potassium, magnesium and phosphorus are shown in Table 24, 25, 26, and 27. As might be expected there were significant negative balances for phosphorus and potassium during the protein-free diet period, probably caused by breakdown of body tissues. All the subjects were in balance during the medium-protein diet period, and no further increase in retention of phosphorus was noted during high-protein diet period. The lower urinary and fecal phosphorus during the high-protein diet period might be partly a result of lower intake of

Table 24 Sodium Intake, Excretion and Balance

Subject	Diet	Order	Sodium Intake[a]	Urinary Sodium[b]		Fecal Sodium[c]	Sweat Sodium[d]	Apparent Balance[e]
			gm/day					
				mean±S.D.				
1	0.9N	2	3.46	3.42	0.38	0.04	0.07	-0.07
	12N[f]	3	3.05	2.98	0.30	0.01	0.09	-0.03
	12N[f]	4	3.05	2.80	0.21	0.02	0.10	+0.13
	24N	1	3.11	3.14	0.38	0.01	0.11	-0.15
2	0.9N	1	3.46	3.85	0.53	0.07	0.20	-0.66
	12N[f]	3	3.05	2.98	0.06	0.04	0.15	-0.12
	12N[f]	4	3.05	2.59	0.17	0.07	0.19	+0.20
	24N	2	3.11	2.85	0.23	0.06	0.04	+0.16
3	0.9N	1	3.46	3.80	0.46	0.05	0.08	-0.47
	12N	2	3.05	2.69	0.38	0.03	0.04	+0.29
	24N	3	3.11	3.18	0.23	0.01	0.09	-0.17
	12N+Ca	4	3.05	2.67	0.17	0.01	0.12	+0.25
4	0.9N	3	3.46	3.52	0.47	0.07	0.10	-0.23
	12N	2	3.05	2.79	0.52	0.07	0.08	+0.11
	24N	1	3.11	3.34	0.26	0.02	0.11	-0.36
	12N+Ca	4	3.05	2.85	0.24	0.05	0.12	+0.03
5	0.9N	3	3.46	3.48	0.51	0.05	0.23	-0.30
	12N	1	3.05	2.93	0.16	0.05	0.23	-0.16
	24N	2	3.11	3.03	0.23	0.03	0.17	-0.12
	12N+Ca	4	3.05	2.31	0.41	0.02	0.18	+0.54
6	0.9N	2	3.46	3.39	0.34	0.09	0.04	-0.06
	12N	1	3.05	3.19	0.42	0.08	0.21	-0.43
	24N	3	3.11	2.86	0.19	0.03	0.14	+0.08
	12N+Ca	4	3.05	2.54	0.17	0.04	0.15	+0.32
						mean±S.D.	mean±S.D.	mean±S.D.
Mean[h]	0.9N			3.58	0.20	0.06 0.02	0.12 0.08	-0.30 0.23
	12N			2.93	0.17	0.05 0.03	0.13 0.08	-0.06 0.25
				(2.90	0.22	0.06 0.02	0.14 0.09	-0.05 0.32)[i]
	24N			3.07	0.19	0.03 0.02	0.11 0.04	-0.09 0.19
	12N+Ca			(2.60	0.23	0.03 0.02	0.14 0.03	+0.29 0.21)[i]

[a]Based on analysis of composites

b,c,d,e,f,h,i, Same as footnotes of Table 23

Table 25 Potassium Intake, Excretion and Balance

Subject	Diet	Order	Intake[a]	Urinary Potassium[b]	Fecal Potassium[c]	Sweat Potassium[d]	Apparent Balance[e]
				gm/day			
				mean±S.D.			
1	0.9N	2	3.11	3.04 0.15	0.33	0.07	-0.33
	12N$_f$	3	3.18	2.49 0.14	0.31	0.09	+0.29
	12N[f]	4	3.18	2.27 0.35	0.34	0.09	+0.48
	24N	1	2.73	2.20 0.18	0.15	0.12	+0.26
2	0.9N	1	3.11	2.55 0.19	0.67	0.12	-0.23
	12N$_f$	3	3.18	2.21 0.17	0.45	0.17	+0.35
	12N[f]	4	3.18	1.97 0.06	0.59	0.18	+0.44
	24N	2	2.73	1.70 0.19	0.20	0.04	+0.79
3	0.9N	1	3.11	2.59 0.54	0.60	0.09	-0.17
	12N	2	3.18	1.92 0.13	0.60	0.04	+0.62
	24N	3	2.73	2.01 0.09	0.28	0.16	+0.28
	12N+Ca	4	3.18	2.10 0.20	0.43	0.17	+0.48
4	0.9N	3	3.11	2.84 0.29	0.43	0.07	-0.23
	12N	2	3.18	2.28 0.14	0.46	0.08	+0.36
	24N	1	2.73	1.74 0.26	0.16	0.09	+0.74
	12N+Ca	4	3.18	2.25 0.18	0.46	0.08	+0.39
5	0.9N	3	3.11	2.85 0.41	0.52	0.07	-0.33
	12N	1	3.18	1.92 0.15	0.58	0.08	+0.60
	24N	2	2.73	1.46 0.17	0.33	0.07	+0.45
	12N+Ca	4	3.18	2.07 0.15	0.59	0.08	+0.44
6	0.9N	2	3.11	2.68 0.18	0.68	0.01	-0.26
	12N	1	3.18	2.30 0.27	0.71	0.11	+0.06
	24N	3	2.73	1.46 0.17	0.51	0.08	+0.68
	12N+Ca	4	3.11	2.10 0.18	0.64	0.08	+0.36
					mean±S.D.	mean±S.D.	mean±S.D.
Mean[h]	0.9N			2.76 0.19	0.54 0.14	0.07 0.04	-0.26 0.06
	12N			2.18 0.25	0.52 0.14	0.10 0.04	+0.38 0.21
				(2.11 0.21	0.59 0.10	0.08 0.03	+0.41 0.26)[i]
	24N			1.83 0.26	0.27 0.14	0.09 0.04	+0.53 0.23
	12N+Ca			(2.11 0.10	0.53 0.10	0.10 0.05	+0.42 0.05)[i]

[a]Based on analysis of composites

b,c,d,e,f,h,i, Same as footnotes of Table 23

Table 26 Magnesium Intake, Excretion and Balance

Subject	Diet	Order	Intake	Urinary Magnesium[a]	Fecal Magnesium[b]	Sweat Magnesium[c]	Apparent Balance[d]
				mg/day			
				mean±S.D.			
1	0.9N	2	664	163 10	458	3	+40
	12N[e]	3	649	173 20	448	2	+26
	12N[f]	4	649	197 6	474	3	-25
	24N	1	604	244 49	336	4	+20
2	0.9N	1	664	166 20	428	4	+66
	12N[e]	3	649	162 20	433	3	+51
	12N[f]	4	649	151 8	499	3	- 4
	24N	2	604	177 17	440	1	-14
3	0.9N	1	664	123 8	535	3	+ 3
	12N	2	649	131 25	542	1	-25
	24N	3	604	141 14	484	3	-24
	12N+Ca	4	649	125 9	536	2	-14
4	0.9N	3	664	115 5	535	2	+12
	12N	2	649	128 30	539	1	-19
	24N	1	604	170 1	448	3	-17
	12N+Ca	4	649	134 5	524	2	-11
5	0.9N	3	664	132 18	596	2	-66
	12N	1	649	126 6	468	5	+50
	24N	2	604	118 14	657?	3	-174?
	12N	4	649	113 15	505	2	+29
6	0.9N	2	664	137 12	538	1	-12
	12N	1	649	166 12	484	5	- 6
	24N	3	604	141 23	473	2	-12
	12N+Ca	4	649	142 15	496	2	+ 9
					mean±S.D.	mean±S.D.	mean±S.D.
Mean[h]	0.9N			139 21	515 61	3 1	+ 7 45
	12N			148 22	486 66	3 2	+13 34
				(138 19	508 38	3 2	0 34)[i]
	24N			165 44	436 59[g]	3 1	- 9 17[g]
	12N+Ca			(129 12	513 18	2 0	+ 3 20)[i]

[a]Based on analysis of composites

[b,c,d,e,f,h,i]Same as footnotes of Table 23

[g]Mean and standard deviation of average of subjects 1,2,3,4 and 6(subject 5, excluded)

Table 27 Phosphorus Intake, Excretion and Balance

Subject	Diet	Order	Intake[a]	Urinary Phosphorus[b]	Fecal Phosphorus[c]	Apparent Balance[g]
					gm/day	
				mean±S.D.		
1	0.9N	2	2.03	1.64 0.15	0.50	-0.11
	$12N_f$	3	2.01	1.54 0.10	0.45	+0.02
	$12N^f$	4	2.01	1.54 0.19	0.42	+0.05
	24N	1	1.67	1.47 0.07	0.38	-0.18
2	0.9N	1	2.03	1.58 0.27	0.67	-0.22
	$12N_f$	3	2.01	1.42 0.07	0.47	+0.12
	$12N^f$	4	2.01	1.34 0.07	0.59	+0.08
	24N	2	1.67	1.19 0.15	0.33	+0.15
3	0.9N	1	2.03	1.45 0.10	0.72	-0.14
	12N	2	2.01	1.20 0.07	0.67	+0.14
	24N	3	1.67	1.10 0.17	0.52	+0.05
	12N+Ca	4	2.01	1.01 0.05	0.75	+0.20
4	0.9N	3	2.03	1.46 0.22	0.76	-0.19
	12N	2	2.01	1.28 0.04	0.56	+0.17
	24N	1	1.67	1.12 0.12	0.40	+0.15
	12N+Ca	4	2.01	1.06 0.03	0.74	+0.21
5	0.9N	3	2.03	1.63 0.11	0.69	-0.29
	12N	1	2.01	1.37 0.10	0.62	+0.02
	24N	2	1.67	1.19 0.11	0.64	+0.16
	12N+Ca	4	2.01	1.02 0.11	0.71	+0.28
6	0.9N	2	2.03	1.44 0.07	0.78	-0.19
	12N	1	2.01	1.35 0.15	0.65	+0.01
	24N	3	1.67	1.05 0.07	0.53	+0.09
	12N+Ca	4	2.01	1.06 0.10	0.82	+0.13
					mean±S.D.	mean±S.D.
Mean[h]	0.9N			1.53 0.07	0.69 0.10	-0.19 0.06
	12N			1.36 0.12	0.57 0.09	+0.08 0.07
				(1.30 0.08	0.63 0.05	+0.09 0.08)[i]
	24N			1.20 0.14	0.47 0.12	+0.07 0.13
				(1.04 0.03	0.76 0.05	+0.21 0.06)[i]

[a]Based on analysis of composites

b,c,e,f,h,i Same as footnotes of Table 23

phosphorus during the high-protein diet period (Table 27). Calcium supplementation apparently influenced the phosphorus metabolism. The dietary calcium/phosphorus ratio was about 1/20 during the low calcium intake. The ratio increased to 1/2 when 900 mg calcium per day was supplemented during the last period. Although the phosphorus intake was the same for the two periods a marked decrease in urinary phosphorus (average 0.34 gm per day or 25%), an increase in fecal phosphorus (0.13 gm per day or 20%) and an increase in phosphorus retention (0.2 gm per day) were noted in all subjects. Every subject went from a negative balance for potsssium during the protein-free diet period to a positive balance during the medium- and high-protein diet periods. Potassium metabolism did not seem to be affected by calcium supplementation.

The effect of dietary protein level on the other divalent mineral measured, magnesium, was quite different from that of calcium. There was no wide individual variation in urinary magnesium excretion as occured in urinary calcium. The daily urinary magnesium excretion ranged from 113 to 244 mg per day for all the periods in all subjects. Losses in sweat were small (3 mg in average) in comparison to the urinary excretion (150 mg per day). There was no clear correlation between protein intake and either urinary, fecal, sweat magnesium or magnesium balance.

Calcium supplementation did not result in any consistent change in magnesium excretion and balance.

Every subject had a negative sodium balance during the protein-free diet period due to higher urinary sodium excretion. The dietary protein in both the medium- and high-protein diet periods tended to decrease the negative balance or to convert it to a positive balance. During the last period, there was an increase in sodium retention which did not appear to be related to the added calcium since it occurred for all subjects not just the four receiving calcium supplementation (Table 24).

DISCUSSION

The most significant result observed in the first three periods of this study was the change in urinary calcium excretion. The urinary calcium increased as protein intake increased. Dietary components other than protein and carbohydrate were approximately equalized and adequate vitamins and trace minerals were provided. It is still possible but unlikely that some unknown factor or factors, such as might exist im meat, increased as the protein level in the diet increased. However, this is unlikely based on other experiments performed in our laboratory.

Previous studies in this laboratory using purified protein formulas demonstrated a similar response to protein intake. When protein intake were varied from protein-free to 600 gm per day, a direct relationship between urinary calcium and protein intake was observed in every subject regardless of the protein sources[1,2,3]. It is rather unlikely that there would be some unknown factor or factors common to all the different source of protein used in the formula diets which would change in proportion to the level of protein. Moreover, using different patterns of crystalline amino acids, Weller (194) demonstrated that the urimary calcium increased as the levels of total nitrogen from amino acids increased. The changes, however, were not related to differences in

the amino acid patterns.

Increase in urinary calcium excretion can result from one or more of the following mechanisms: increase in the filtered load of calcium in glomerular filtrate, increase in renal tubular calcium secretion, or decrease in renal tubular calcium reabsorption.

The plasma concentration of calcium in fasting blood samples taken at the middle and at the end of each period did not show any significant change related to protein and/or calcium intake. Diurnal change in plasma calcium has been shown to be only about ± 3% in spite of large variations in calcium intake and urinary calcium excretion (119). Fraction of filtrable calcium in plasma has also been shown not to be affected by the diet (57). Therefore, the concentration of calcium in glomerular filtrate would not be expected to vary to any great extent. McFadyen et al. (195), however, correlated a slight decrease in plasma calcium and filtrable calcium levels with urinary calcium excretion. The discrepancy between the results of McFadyen et al. and our results possibly may be explained by the length of experiments. They measured plasma concentration of calcium only on the second and third day after initiation of a low calcium diet.

The filtered load of calcium may also be modified by a change in the glomerular filtration rate (GFR). GFR

has been shown to be related to protein intake (see page 33-35 for rev. of literature). The rate of creatinine clearance calculated for the present experiment was markedly increased during the high-protein intake. Because creatine in the meat is readily converted to creatinine on heating (178), the rise in creatinine clearance rate during the high-protein diet period was partly the result of increased exogenous creatinine. GFR obtained from these creatinine clearance rates could be overestimated for the the high-protein diet period. Even though the GFR was overestimated during the high-protein intake period in this experiment, 25% increase in GFR during high-protein period (Table 16), could not account for the more than 200% increase in urinary calcium.

Increase in GFR alone has been shown to have little effect on sodium excretion unless the extracellular volume is expanded (55). Although the same kind of study for calcium has not been done, many hemodynamic factors which affect the calcium excretion have been shown to have no effect on GFR (see page 16 for rev.). However, Hodgkinson and Heaton (57) correlated an increase in urinary calcium excretion to a change in GFR. Discrepancy between their study and our study may be due to the differences in the duration of the studies. Hodgkinson and Heaton measured GFR immediately after intake of food whereas in our experiment sufficient time was allowed for

equilibrium on the different diets.

When the glomerular filtration rates for the variable protein intakes are adjusted to a fixed volume of glomerular filtrate (G.F.) of 100 ml, differences in the filtered load of calcium resulted from changes in GFR are eliminated. Calcium excreted per 100 ml G.F. during the higher protein intake periods are still markedly increased. Alteration in filtered load is not the main mechanism responsible for calciuretic effect of high protein intake.

Bidirectional flux of calcium has been shown to exist in the renal tubules (54, 67, 69). Frick et al. (67) also demonstrated in rats that the net calcium influx into the proximal tubules occurs during microperfusion with a calcium-free solution. However, they noted no further increase in calcium concentraion in the perfusate in the distal segments of the tubules if the concentration of calcium reached 2.11 mEq./l. In the present study, the urinary calcium concentration in most subjects was more than 2.66 mEq./l, but variation in protein intake still exerted a remarkable effect on calcium excretion. It also affected those who had high calcium concentration in urine. It is very unlikely that the calciuretic effect occurs by enhancement of tubular calcium secretion. A change in the tubular reabsorption, therefore, is most likely the principle

mechanism involved in the calcium excretion related to protein intake.

There are many factors which can affect tubular calcium reabsorption. Osmotic pressure changes induced by urea have been shown by many investigators to affect urinary calcium excretion (5, 54, 71 for rev.). However, other experiments have not shown this effect (53, 196, 197). Figure 1a-f show that both urinary nitrogen and calcium increased as dietary protein increased. These two urinary components, however, were not correlated between the subjects or even between various periods for the same individual. Infusion of urea equivalent to the amount produced by the high protein diet also did not result in significant increase in urinary calcium[3].

Although high protein intake has been shown to cause increase in blood pressure in experimental hypertensive rats (198), this effect was not observed in our subjects. Extracellular space also was not increased by protein intake (55). Therefore, a decrease in reabsorption of calcium in the tubules caused by hemodynamics factors is also high unlikely.

Correlation between urinary calcium excretion and excretion of sodium, magnesium and other minerals has been observed (see page 15 for rev.). This relationship was not observed during periods of variable protein intake. It suggests that some other specific mechanisms

for calcium reabsorption in tubules neither common nor coupling to the reabsorption mechanisms of other minerals exists. It is very possible this specific calcium reabsorptive system can be affected greatly by variations in protein intake but other transport systems are either not or only slightly affected.

Katz and Epstein (182) correlated the increase in sodium reabsorption to the increased activity of the specific enzyme, sodium-potassium-stimulated ATPase during the high protein intake period. Although there was no similar study on the activity of enzymes involved in calcium reabsorption, it is possible that a high protein intake may inhibit activity of enzymes involved in calcium reabsorption but have a minimum or a different effect on the enzymes involved in the systems for other minerals.

In contrast to the kidney, calcium excretion by sweat glands was not affected by variations in protein or calcium intake. The dermal nitrogen excretion, however, increased as protein intake increased. This difference between calcium and nitrogen most likely was a consequence of difference in plasma levels of calcium and urea. Plasma concentration of urea were increased during higher protein intake, but plasma calcium was not affected by either protein or calcium intake. A good correlation between plasma urea level and nitrogen in sweat was

Sirbu et al. (189) and Costa et al. (51).

The average daily loss of calcium in sweat was about 15 mg per day in this study. This value is approximately the same as that estimated by Leitch and Aitken (46), Gitelman and Lutwak[5], McKay et al.[6]; however, it is much lower than that obtained in a study by Consolazio et al. (48). They estimated a loss 3 mg per hour or 72 mg per day for subjects resting under comfortable conditions. The difference possibly resulted from the method of study. They collected sweat from arm bands rather than determing total body sweat as was done in this study. Sweat collected from arm bands has been shown to be more concentrated for many substances (89,199). A later report from Consolazio et al. (200) also showed that the concentration of calcium in arm sweat was slightly higher than total body sweat. The lengh of interval of collection was also different between our study and that of Consolazio et al. (48). They apparently neglected the factor of acclimatization since sweat was collected over a short interval. The volume of sweat as well as the concentration of calcium has been shown to decline dramatically in a period of few hours (49, 52). Thus, the values of Consolazio et al. (48) was possible overestimated.

Although the daily loss of calcium in sweat is small, this amount should not be neglected. It becomes

a relatively more important route for calcium loss when the urinary calcium excretion decreases. In the present study, more calcium was excreted in sweat than in urine in one period by one subject. Calcium loss in sweat also increased greatly following strenous physical activity even during the low calcium intake periods. These findings emphasize that calcium loss in sweat must be taken into consideration in calcium balance studies, in kinetics studies and in establishing requirement for calcium intake.

In addition to changes in urinary calcium excretion, intestinal calcium absorption can also be affected by a low calcium and variable protein intake. The average factional absorption was 70% during the low calcium intake. This rate is distinctly higher than that previously reported (Table 1). Intestinal calcium absorption was also shown to be enhanced by higher protein intakes.

The amount of calcium in the feces was greater than the ingested calcium during the periods of low calcium intakes except in one period for one subject. From the average fractional absorption rate and net ^{47}calcium absorption study, it appears that only 30 mg of the average fecal calcium (145 mg) was of dietary origin; the rest was endogenous fecal calcium. This indicated that a large amount of endogenous calcium was being

secreted into gut.

A large volume of digestive juice is secreted per day (4-10 l) and the calcium concentration in these fluids varies from 2.1 to 14.4 mg per 100 ml (126). Calculated from these values, the total digestive juice calcium has been estimated to be between 300 to 1100 mg per day (126).

Digestive juice calcium has been considered by some investigators as being a more physiologically meaningful vector than endogenous fecal calcium (17); however, the amount of calcium in digestive juice is difficult to measure. Two of the three calculation made in this study were done according to the method of Aubert et al. (17): $V_d = V_{ef} / (1-\alpha)$. We also used a continuous oral feeding method to assess the digestive juice calcium directly. This method has not been reported previously in the literature for measurement of digestive juice calcium. At the end of seven days of continuous oral feeding of ^{47}calcium, the specific activity of ^{47}calcium in feces and urine almost reached a steady state (117). From the specific activity of ^{47}calcium in dietary, urinary and fecal calcium, the amount of calcium secreted into the gut can calculated. Two subjects who had been measured by this same method in a previous experiment showed almost identical values even with a high calcium intake of approximately 1.5 gm per day. Such reproducibility

indicates this direct method gives a better estimation of digestive juice calcium.

Malm (126) estimated the digestive juice at an average of about 750 mg per day with a range from 400 to 1100 mg. From the results of the fecal calcium and ^{47}calcium study, endogenous fecal calcium was estimated to average about 115 mg per day. The fractional re-absorption rate of digestive juice calcium would be more than 85% if 750 mg calcium was secreted with 115 mg endogenous fecal calcium. This fractional absorption rate of digestive juice calcium would be greater than that for dietary calcium. The value of Malm (126) is probably overestimated.

On the other hand, Heaney and Skillman (19) estimated that the digestive juice calcium is 194 ± 37 mg per day. Almost all values from our studies were much larger than this average. Although no actual values by direct measurements for total digestive juice calcium are available for comparison, from our data of fecal calcium excretion, ratio of specific activity of ^{47}calcium of dietary and fecal calcium (Table 18) and some available data of total calcium in some selected digestive juice (21, 126) the value of Heaney and Skillman would appear to be underestimated.

The results of this study also gave very diverse values in the same individual using two different methods

of calculation. This diversity of calculated values may indicate the inadequacy of our assumptions for calculation. We assumed that the digestive juice was mixed homogenously with dietary calcium in the gut and then both were absorbed at the same rate. Such assumptions may not be valid. Heaney and Skillman (19) considered that part of the digestive juice calcium is secreted into the lower segment of gut where no more calcium absorption takes place. Schedl et al. (20) found that one-third of the digestive juice calcium readily formed calcium phosphate and was precipitated. This precipitated calcium complex, however, may later be dissociated then calcium becomes available again for absorption.

Although the determined amount of digestive juice calcium may not be very accurate, neverless, the large amount the digestive juice calcium can not be disregarded. There is no experimental or clinical evidence in favor of a regulated digestive juice calcium (126). However, this large amount of calcium provide a homeostatic mechanism to prevent large fluctuation of calcium in the gut. The significance of this digestive juice calcium becomes even more important during low calcium intake such as in the present study. The secreted calcium was many times larger than the ingested calcium.

The present study also showed a wide variation of digestive juice calcium between the individuals.

The amount of calcium absorbed from the digestive juice calcium supposedly is also quite varied. On the analysis of the correlation between calcium absorption and other parameters of calcium metabolism, however, Bronner et al. (30), Bronner (5), Malm (43) and Phang et al. (32) neglected this reabsorbed calcium from digestive juice calcium.

Other components in digestive juice besides calcium need to be considered when evaluating calcium absorption. Although a low calcium diet caused a significant increase in the efficiency of calcium absorption. Fromm et al. (201) did not find any enhancement in the efficiency in radiocalcium absorption during fasting even when the calcium in the gut was very low. This may suggest that the digestive juice secreted in response to the stimulation by food greatly enhanced the absorption of calcium. Because an increase in the intake of protein can enhance calcium absorption, the protein content of digestive juice may be a very important factor involved in modifying calcium absorption. Nasset and his colleagues (148 for rev.) have shown that the gut contributes a large amount of protein from digestive juice and shed mucosa cells in order to prevent a large fluctuation of amino acids in the gut. The significance of a constant molar ratio of different amino acids in the gut in relation to calcium absorption is unknown.

In experimental animals, it has been shown that the protein content of the digestive organs decreases rapidly during a protein free diet. The decrease in endogenous protein from the digestive juice and shed cells would be expected as a consequence of prolonged protein-free diet. Such an effect may have contributed to the decrease in calcium absorption observed during the protein-free diet period.

Bone is the enormous reservoir of calcium. The total negative balance of calcium of 5.1 to 16.3 gm in 45 or 60 days represents only 0.4 to 1.0 % of the total calcium in bone. Two methods have been employed at the end of each period to study the mineral changes in bone (Appendix 4 and 5). At the end of 45 or 60 days, no consistent and significant change was noted using either method. Sorenson and Cameron indicated that at least a 2% error in accuracy and reproducibility existed in their photon bean scanning method. Vose also estimated that the error can be as large as 20% in X-ray densitometric study of bone (202). Both methods are apparently not sensitive and precise enough to detect this small change. Selective calcium loss in bones studied is also not likely.

The plasma alkaline phosphatase can be used as an index of bone matrix formation (99). Although there were marked differences in calcium excretion and balance

in various periods in the same individual, no significant differences in plasma aklaine phosphatase activity were observed in same individual by variation either in protein or calcium intake. This finding suggests that either the measurement of this enzyme activity was not sensitive enough to detect the change in bone matrix formation or else bone matrix formation was not altered by the different diets.

On the other hand, urinary hydroxyproline is a good index of the rate of breakdown of bone collagen (102, 103). In the present study, urinary hydroxyproline increased markedly as the protein intake increased. Schöfelder (203) measured the urinary hydroxyproline excretion at three levels of protein intake -- 12, 48, 96 gm nitrogen per day. There was marked increase in the urinary calcium with high protein intake. She did not find significant differences in urinary hydroxyproline between the 3 levels of protein intake if an hydroxyproline-free formula was used. However, if turkey and shrimps were substituted for the egg albumin and casein as the protein source, she found a large increase in urinary hydroxyproline with essentially the same effect of protein on calcium excretion. Some subjects who had participated in previous experiments in this laboratory in which formula diets with different levels of protein were used also excreted approximately the same amount of hydroxyproline as was

found during the protein-free period of the present experiment. In the present study, good correlation was noted between rate of calcium leaving the bone and urinary hydroxyproline of protein-free diet period but not between rate of calcium leaving bone and urinary hydroxyproline during period 2 (Table 22). All these findings indicated that the increase in urinary hydroxyproline excretion during the higher protein intake periods resulted from a high content of hydroxyproline in the diet rather than effect of protein intake on the metabolism of bone collagen.

In contrast to the alkaline phosphatase and urinary hydroxyproline excretion, radiocalcium studies provided more information on calcium metabolism in bones.

The miscible pool size of calcium of the subjects in the present study varied from 2724 to 6505 mg (Table 19). These values are similar but slight less in some subjects as compared to values of limited numbers of normal subjects of Bronner et al. (30) and Heaney (204). The higher "corrected" specific activity of urinary ^{47}calcium on the first day after injection (Figure 3) also suggested a smaller pool size. This study was carried out 3-4 weeks after initiation of a very low calcium intake. The miscible pool size of calcium under this circumstances might well be affected. Although many studies have been done on the assumption that the

calcium miscible pool size is not affected when calcium intake varied between 200-2000 mg per day (32), Heaney (204) and Bronner et al. (30) have shown significant decrease in pool size in patients with chronic disuse osteoporosis and postmenopausal osteoporosis.

Marked differences in the time course of pool ^{47}calcium specific activity between the subjects of different protein intake may be more significant. Both subjects 3 and 4 were studied during medium-protein diet period. Both had smaller turnover of calcium pool than the others but subject 4 had a definite decrease in the slope after day 3. Phang et al. (32) also observed a curve similar to the one for subject 4 when their subjects consumed a low calcium intake of 200 mg per day. This decrease in the slope for subject 4 but not for subject 3 may be explained by the dramatic decrease in calcium excretion for subject 4 when he consumed a low calcium diet. The difference in urimary calcium excretion between 2 periods of medium-protein diet with or without calcium supplementation was more than 100 mg per day for subject 4 compared to less than 30 mg for subject 3 (Table 12).

The dilution of tracer proceeded more rapidly for subjects 1, 2, 5 and 6. Comparison of the calcium kinetics data of the present study with that of the normal subjects of Bronner et al (30) and Heaney (204) shows that the rates of calcium entering and leaving bone were higher

than the range for a limited number of their normal subjects, but the pool size was slightly decreased in some subjects. Although the urinary and fecal excretion was decreased during the low calcium intake in this experiment, the amount of decrease in excretion is much less than the amount of increase in rate of calcium entering the bone. Turnover rate is determined by pool size and calcium in urine, feces, sweat and entering bone (i.e. $m = V_T / P$, $V_T = V_u + V_{ef} + V_s + V_{o+}$, see page 52-53). Because of the decrease in pool size and increase in V_T, the turnover rate is expected to increase in these subjects.

Because the subjects had either high-protein (subject 2 and 5) or protein-free (subjects 1 and 6) diets, further investigation is needed to determine whether these findings were the result of interindividual variation or the effect of protein intake. Comparing the similar time course curves of specific activity of pool ^{47}calcium after cessation of oral calcium (Figure 4), there was little or no difference between the subjects after a medium-protein diet with or without calcium supplementation. The changes in turnover rate may be partly due to the effect of protein intake.

The mechanism of increase in turnover during very low calcium intake with either high-protein or protein-free diet has not been studied. Increase in the negative

calcium balance during high-protein and protein-free diet periods may be one of the inducing factors. Because there is a regulatory mechanism for coupling processes between the calcium entering and leaving the bone (5, 30, 104), an increase in negative calcium balance during both high-protein and protein-free diet periods could cause an increase in calcium turnover in bone. There is no evidence to show that the metabolism of bone collagen is changed by variation in protein intake. However, dietary variations has been shown to induce structural changes in collagen in rat^{14} (96). Such change in collagen in turn may cause alteration in rate or stability of the interaction between calcium and collagen. Decrease in calcium intake may be an additional important factor. El-Maraghi et al. (169) considered that insufficient calcium may stimulate the resorption of existing bone in order to provide necessary minerals for mineralization of bone matrix.

A linear relationship has been demonstrated between intestinal calcium absorption and rate of calcium entering bone (30, 32); between intestinal calcium absorption and urinary calcium (32, 43); between rates of calcium entering and leaving bone (5, 30, 104); between urinary calcium and endogenous fecal calcium (32); and between intestinal calcium absorption and rate of calcium leaving bone (32). However, it has not been

demonstrated which one of these rates is the independent variable. Any one of these variables may be considered as an independent or dependent variable. In addition, it is also possible that all these relationships are functions of some unknown independent factor or factors. Our data provide some information from which speculation can be made regarding which of the effects on kidney, gut or bone is of primary importance in the calciuretic effect of high protein intake.

Because of very low intake of calcium there was almost no difference in the amount of dietary calcium absorbed in different periods by the same individual. However, there was still a wide variation in urinary calcium excretion in different periods. Moreover, this study shows an increase in urinary calcium excretion but a decrease in endogenous fecal calcium during the high protein intake. These two findings are contradictory to a common regulatory mechanism such as described by Phang et al. (32). Their hypothesis of a common regulatory mechanism for gut, kidney, and bone was not valid under the present experimental condition.

The results of this experiment show that the calciutetic effect of a high protein intake is not necessarily a result of an increase in intestinal calcium absorption. It resulted primarily from the inhibition of renal tubular reabsorption of calcium during high

protein intake. Increase in the amount of dietary calcium absorbed may augument this effect. The effects on calcium metabolism in bone are likely secondary to the calcium balance of the body, but the possibility that these effects resulted primarily from alteration of bone metabolism by variable protein intake can not be ruled out.

A wide variation in pattern and magnitude of responses by the gut, kidney and bone are demonstrated in this experiment. An increase in protein intake from 0.9 to 12 gm nitrogen per day caused a dramatic increase in urinary calcium on subjects 1 and 3 but had little effect on subjects 5 and 6. However, a further increase in protein intake to 24 gm resulted in large increase in subjects 5 and 6 but almost no increase for subject 1 (Table 12). "Sensitivity" of urinary calcium excretion to variation in protein intake apparently differs between individuals.

On the other hand, increase in protein from 0.9 gm to 12 gm or 24 gm nitrogen per day caused more than 200 or 500 % increase in urinary calcium excretion for subject 3. The actual amount of difference in urinary calcium, however, was smaller than that of fecal calcium (Table 12). This indicated that the gut may be more important for certain individuals in the regulation of calcium balance. Individual differences in the responses

of the gut and kidney to low calcium intake have been observed by some investigators (see page 23-26). Such differences in individual responses may partly explain the contradictory results found for the effect of protein intake on calcium metabolism (Table 3).

The effect of variable protein intake on calcium balance during low calcium intake also was a significant result observed in this experiment. The least negative calcium balance was found in 5 subjects during the medium protein intake period. Although there were slight difference in calcium intake during the different periods in this study. The magnitude of change in fecal and urinary calcium in most cases was larger than the differences in calcium intake. It is not likely that the differences in calcium balance resulted primarily from the differences in calcium intake. The large negative balance during the high-protein or protein-free diet periods resulted mainly from an increase in urinary or fecal calcium excretion.

Only a few investigations have been reported using a diet approaching this level of calcium intake. Bauer et al. (132) tried to approximate an "endogenous calcium excretion" with a low calcium intake averaging 110 mg per day. All 27 subjects were in negative balance and daily "endogenous calcium excretion" was about 3.9 mg per kg body weight per day. Other studies on the eva-

lution of special diets such as a maize diet (206), pure meat diet (133) and Taro diet (128) also had very low calcium intake between 80-110 mg per day. Protein intake was not well controlled in these experiments. Large negative balance resuted from increase in urinary calcium excretion in the subject who had a high protein intake of pure meat diet (133).

Our observations are also in consistent with the results of McKay et al. (207) and Johnson et al. (167). MeKay et al noted that the group which consumed the experimental diets retained more calcium than the the group which consumed self-chosen diets at the same calcium intake. The experimental diet had more consistent protein intake, 8.85-13.51 gm nitrogen per day, whereas the self-chosen diets ranged between 5.55 to 16.27gm nitrogen per day. Johnson et al. found an increase in protein intake from 48 to 141 gm per day caused an increment of 163 gm daily in urinary calcium but an increment of only 69 mg in apparent absorption. This resulted a sustantial negative balance on high protein intake even at calcium intake of 1400 mg per day.

The effect of variation in protein intake on calcium requirement has been neglected in many studies conducted to assess calcium requirement (3, 126). Bauer et al. (132) attempted to estimated requirements by measuring the "endogenous calcium excretion". However,

there was about a 25% difference in the "endogenous calcium excretion" between the medium- and high-protein periods in the present study. High protein diet with a low calcium intake is not uncommon in this country. This study demonstrates that the level of protein must be taken into consideration when making recommendations for calcium requirement.

The significance of low calcium and high protein intake on the development of osteoporosis also should be emphazised. Osteoporosis resulting from a high protein and low calcium intake has been demonstrated in experimental animals (169-171). The results of the present experiment suggest that there is the same tendency for adult men. A negative balance of 50 mg per day continued for 30 years would result a total loss of 550 gm calcium, that is, of more than one-third of the total calcium in the body. Moreover, only a slightly positive balance was achieved by calcium supplementation after a long period of negative calcium balance.

SUMMARY

Six healthy young males were confined to a metabolic unit for 60 days. During the first three periods of 15 days each, they consumed three controlled diets containing about 0.1 gm calcium per day at three levels of protein intake: 0.9 (protein-free), 12 (medium-protein) and 24 (high-protein) gm nitrogen per day. Four of the subjects were given 0.9 gm calcium supplements per day while at medium-protein intake during the last 15 days.

This experiment attempted to study the effect of protein intake on calcium metabolism at very low calcium intake and to eludicate the mechanisms of the calciuretic effect of high protein intake.

There was a wide variation in calcium metabolism between individuals. Average daily urinary calcium increase from 51 mg on protein-free diet to 99 mg on medium-protein diet and 161 mg on high-protein diet. A 0.9 gm calcium supplement per day at medium-protein diet increased the daily urinary calcium of 4 subjects from 68 mg to 160 mg. Average daily fecal calcium was 174, 133, 128 mg on protein-free, medium-protein, high-protein diets, respectively. Usual sweat loss of calcium remained at about 15 mg per day regardless of the protein and calcium intakes. Five subjects had the least negative calcium balance during medium-protein diet period. Plasma

calcium and alkaline phosphatase remained unchanged throughout the experiment. During strenous exercise, sweat loss of calcium increased to 25 mg in a 40-minute interval.

The ^{47}calcium study showed an average 70% fractional absorption rate during the very low calcium intake. The increase in protein intake tended to enhance the fractional absorption rate. The miscible pool of calcium was 2724 to 6506 mg. The turnover rate of the calcium pool and the calculated rates of calcium entering or leaving bone were higher on the subjects on protein-free and high-protein diets. Estimated calcium secreted into the gut varied considerably depending on the method. A method of continuous low dose radiocalcium feeding seemed to be the best. 206 - 1135 mg per day for endogenously secreted calcium was estimated using this method.

Our data demonstrated that decrease in renal tubular reabsorption of calcium is responsible for the calciuretic effect of the high protein intake. Inhibition of a specific reabsorptive process in the tubular cells presumably through the inhibition of involved enzymes was postulated as mechanism to explain the decrease in calcium reabsorption. Increase in urinary calcium is not necessarily a result of enhancement of intestinal calcium absorption, but increase in the amount of dietary calcium absorbed may augument this effect.

This study suggested that protein intake must be taken into consideration when making the recommendation for calcium requirements or allowances. There may also be a tendency to develop osteoporosis after long period on low calcium intake especially with a concurrent high protein intake.

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FOOTNOTE

1. Margen, S., and D. H. Calloway, Effect of dietary protein on urinary calcium. Federation Proc., 26: 629, 1967 (abstract).

2. Margen, S., and D. H. Calloway, Effect of high protein intake on urinary calcium, magnesium and phosphorus. Federation Proc., 27: 726, 1968 (abstract).

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14. P. E. McClain, Human Nutrition Research Division, U. S. D. A., A. R. S., Beltsville, Maryland., Seminar given in Department of Nutritional Sciences, University of California, Berkeley, 1970.

15. Life Saver and Orange Slice, both essentially calcium and nitrogen free.

16. By Osmette (Precision Osmometer), Precision Systems, Framingham, Massachusetts.

17. By Combistix, Ames Co., Elkhart, Indiana.

18. This kind of counting was used for the intravenous ^{47}Ca. study because of the very low radioactivity in the feces. The calcium oxalate in slurry would not precipitated and therefore would give approximately the same geometry as calcium oxalate dissolved in the solution. The recovery rate of radioactivity of this method was 94-105 %.

Appendix 1.

Procedures for Preparation of Urinary Samples for Radioactivity Determination :

1. Acidify the urine to pH 1 using conc. HCl solution.
2. Put the flasks on a steam bath for about 15 min. to solubilize the calcium phosphates.
3. Remove the flasks from bath and add 10 ml 2.5 % oxalic acid per 100 ml urine.
4. Add conc. NH_4OH to bring pH to 4.8-5.3 using bromocresol green as indicator.
5. Let stand overnight to allow calcium oxalate precipitate to settle.
6. Decant or siphon off the supernatant after the precipitate has settled.
7. Transfer the precipitate to a centrifuge tube and wash the precipitate twice with a 2.5% oxalic acid solution.
8. Add approximately 10 ml or more concentrated HCl solution to the tube, shake and stir.
9. Centrifuge the tube again and transfer the supernatant to a counting tube and adjust the final volume to 10 ml with conc. HCl.
10. After determination of radioactivity, dilute the solution in counting tube to an appropriate volume with 1 N HCl solution and determine the calcium content by atomic absorption spectrophotometry.

Appendix 2.

Procedures of Preparation Fecal Samples for Radioactivity Determination:

1. Weigh the bucket to determine the weight of feces.
2. To the feces add 2 N HCl solution in a ratio of 2 or more parts of HCl solution to 1 part of feces.
3. Homogenize the feces and HCl solution using the polytron, keep an aliquot of homogenate for calcium determination (for daily fecal calcium).
4. Add enough concentrated HCl solution to the homogenate to bring pH to 1 using methyl red as an indicator.
5. To the homogenate add twice its volume of acetone and filter through a coarse filter paper (No. 515, Eaton-Dikeman Co.).
6. To the filtrate, add 10 ml of 2.5% oxalic acid per 100 ml filtrate.
7. Add conc. NH_4OH solutiion to the filtrate and bring the pH of the filtrate to pH 4.8-5.3 using bromocresol green as indicator.
8. Let stand over night for calcium oxalate precipitate to settle.
9. Decant or siphon off the supernatant after the precipitate has settled.
10. Transfer the precipitate to centrifuge bottles and wash the precipitate twice with 2.5% oxalic acid.
11. Add few ml of oxalic acid to the washed precipitate to make a slurry and transfer directly to a counting tube for radioactivity determination. The slurry is then washed into a large volumetric flask with conc. HCl solution and filtered. Diluted the filtrate to an optimal concentration for calcium determination.
12. Alternatively, add 20-60 ml conc. HCl solution to the slurry to dissolve the calcium oxalate, transfer the clear supernatant solution after centrifuge to a counting tube and adjust the final volume to 10 ml. Radioactivity and calcium content in the tube is determined using the same method as for the urinary preparation.

Appendix 3

Assumption and Derivation of the Equations

1. Equation 1

Because endogenous fecal calcium and urinary calcium are considered to come from same pool of extracellular fluid, the specific activity of endogenous fecal calcium and urinary calcium are the same,

$$S.A._{ecf} = \frac{R_{ef}}{V_{ef}} = \frac{R_u}{V_u} \quad , \text{ and } \quad V_{ef} = \frac{R_{ef} \times V_u}{R_u}$$

R_{ef} equals R_F if radiocalcium is given intravenously.

2. Equation 7

Because the ingested calcium is assumed to be mixed homogenously with digestive juice calcium and both absorbed at the same rate, specific activity of fecal calcium is the same as the specific activity of calcium in the intestine,

$$S.A._F = \frac{V_d \times S.A._{ecf} + R_i}{V_d + V_i} = \frac{V_d \times S.A._u + R_i}{V_d + V_i}$$

Rearangement of the above equation gives equation 7.

3. Equation 8

a) $\frac{R_u}{V_u} = \frac{R_{ef}}{V_{ef}}$

b) $\alpha = \frac{V_i + V_{ef} - V_F}{V_i} = \frac{R_i + R_{ef} - R_F}{R_i}$

c) $R_{ef} = \frac{R_u}{V_u} \times V_{ef}$, from a)

Substitute c) in equation b) for R_{ef}. Rearrangement the substituted equation gives equation 8

Appendix 4

X-Ray Aluminum Equivalencies for Phalanx 5-2 at End of Each Period[a]

Period	Subject					
	1	2	3	4	5	6
			cm			
On admission	.223	.222	.201	.231	.207	.224
1st	.214	.217	.194	.222	.193	.218
2nd	.220	.232	.207	.228	.207	.224
3rd	.222	.231	.204	.218	.195	.225
4th	.222	.226	.201	.219	.200	.215

[a]Courtesy of Dr. G.P. Vose. Roentgenograms were taken in Berkeley under the instruction of Dr. Vose and then sent to Texas Women University, Denton, Texas for evaluation. The method of radiographic densitometry have been reported elsewhere (202, 207).

Appendix 5

Mineral Content of Distal Left Radius[a]

Period	Subject					
	1	2	3	4	5	6
	gm/cm					
on admission	1.23	1.03	0.82	1.12	1.05	1.66
1st	1.34	0.97	0.84	1.13	0.75	1.63
2nd	1.30	1.05	0.86	1.22	0.97	1.63
3rd	1.40	1.10	0.85	1.18	0.98	1.59
4th	1.38	1.13	0.88	1.19	0.94	1.61

[a]Courtesy of Dr. N.F. Goldsmith. Mineral Content was measured by her using the method of Sorenson and Cameron (111) at the end of each period.