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Human Bone Collagen Synthesis Is a Rapid, Nutritionally Modulated Process

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ABSTRACT: We developed a direct assay of human bone collagen synthesis using [^{13}C] or [^{15}N] proline and applied it to determine the effects of feeding in young healthy men. Surprisingly, postabsorptive bone collagen synthesis is not sluggish, being ~0.07%/h more rapid than that of muscle protein, and capable of being stimulated within 4 h of intravenous feeding by 66 ± 13%.

Introduction: All current methods for estimation of bone collagen turnover are indirect, depending on the assay of collagen "markers." Our aim was to develop a direct method for human bone collagen synthesis to be used to study its physiology and pathology, and specifically, in the first instance, the effect of feeding.
Materials and Methods: We applied, over 2 h, flooding doses of [^{13}C] and [^{15}N] proline to label iliac crest bone collagen in eight young healthy men. The rate of collagen synthesis was determined as the rate of labeling of collagen hydroxyproline (assayed by gas chromatography–combustion–isotope ratio mass spectrometry in collagen extracted by differential solubility) compared with plasma proline labeling (assayed by gas chromatography–mass spectrometry). We also determined (in a second group of eight young healthy men) the effect of intravenous nutrition (glucose, lipid emulsion, and amino acids (in the ratio of 55%:30%:15% energy, respectively).
Results: Free bone proline labeling was 92 ± 6% of that of plasma proline, supporting the flooding dose assumption. Human iliac crest bone collagen is heterogeneous, with NaCl-EDTA, 0.5 M acetic acid, pepsin-acetic acid, and hot water–extractable pools being responsible for ~1%, 3%, 8%, and 81% of content, respectively. The synthetic rates were 0.58 ± 0.1, 0.24 ± 0.05, 0.07 ± 0.02, and 0.06 ± 0.01%/h, respectively, giving an average rate of ~0.066%/h. [^{13}C] and [^{15}N] proline gave identical results. Intravenous nutrition caused the disappearance of proline label from the procollagen pool and its increased appearance in the less extractable pools, suggesting nutritional stimulation of collagen processing.
Conclusion: The results show (1) that iliac crest bone collagen synthesis is faster than generally assumed and of the same order as muscle protein turnover and (2) that feeding increases synthesis by ~66%. Given its ability to detect physiologically meaningful responses, the method should provide a new approach to studying the regulation of bone collagen turnover.
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Key words: metabolism, matrix protein, stable-isotope tracer, proline, bone turnover, nutrition

INTRODUCTION

The thinning and wasting of bone are major clinical problems that affect many people in later life, particularly postmenopausal women, and are associated with increased risk of fracture and a decreased quality of life. There is increasing evidence that links optimal nutrition to bone health and protein undernutrition to an inability to achieve peak bone mass during growth or maintain it, leading to increased fracture risk in later life[1–3] However, our understanding of the mechanisms underlying bone diseases is hampered by a lack of knowledge of the normal short-term physiology of human bone collagen metabolism. It is important to understand the physiology of bone collagen in humans as it confers many of bone's useful mechanical properties such as elasticity, resistance to bending, and toughness on impact, which contribute to bone quality.[4,5] Many of the problems associated with the greater risk of fracture in osteopenic individuals are probably at least as much caused by a diminution to bone quality (i.e., architectural and mechanical properties of bone, some of them dependent on the collagen content of bone) as to low bone mass.[4,6] Also, evidence from animal studies suggests that loss of bone mass can be caused as much by derangements in the formative process[7,8] as by increases of resorption.

Although there are a number of indirect methods for

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assessing bone collagen turnover,[9] these have never been validated against direct measures of bone collagen synthesis or breakdown (e.g., as distinct from calcium turnover) in humans. Until recently, the feasibility of making such direct measurements was poor because of the lack of sufficiently sensitive, precise, and accurate methods. Nevertheless, the rate of human skin collagen synthesis has been directly measured by the incorporation of a labeled tracer into protein over time[10] using a primed, constant intravenous infusion of [^{13}C]proline, with the collagen synthetic rate being determined from the ratio between the labeling of dermal collagen and tissue prolyl *t*-RNA. However, collagen synthetic rates were found to be very variable (±75% of the mean), and with physiological processes often producing changes of only 20–60%, this makes the approach impracticable for bone, even if sufficient osteoblasts for the assay of prolyl *t*-RNA could be harvested from the small bone biopsies (~100–200 mg) in an ethically permissible fashion.

Given these limitations, our aim was to develop a convenient, practically robust, sensitive method capable of detecting the effects of a physiological stimulus and use it to study the short-term effects on feeding. We have therefore adopted an alternative approach that uses a "flooding dose"[11,12] (i.e., a large bolus of stable isotopically labeled proline that becomes rapidly distributed through plasma and extracellular and intracellular spaces), so that the labeling in proline in these pools and the prolyl *t*-RNA becomes rapidly equilibrated. By this means, the labeling of the plasma proline, which is easily measured, provides a surrogate for that of bone prolyl *t*-RNA, which cannot be measured directly because of the low abundance of bone osteoblasts and small sample sizes. Furthermore, the technical problems of separating bone collagen from other proteins and of measuring incorporation of proline into relatively impure mixtures can be elegantly overcome by measuring incorporation of labeled proline into hydroxyproline, which is only found in collagen. With current, state-of-the-art, gas chromatography–combustion–mass spectrometry methodology, it seemed feasible to measure collagen synthesis in small samples of bone (~150 mg), both sensitively and precisely.

With the availability of a robust method capable of making repeated measurements of human bone collagen synthesis within a few hours, this should not only provide a tool to study normal human bone physiology, but also to test new approaches to optimizing bone collagen mass in a variety of clinical situations in which bone formation might be increased (e.g., with use of PTH analogs). Here we report novel results showing the rapidity of human bone collagen synthesis, the heterogeneity of synthetic rates of fractions of bone collagen of different degrees of maturity, and their acute responses to feeding.

MATERIALS AND METHODS

Subjects

Preliminary studies using animal and human bone obtained at surgery indicated that a CV for repeated analysis of collagen labeling of a sample from the same individual was ±3%, much less than the likely population variance.

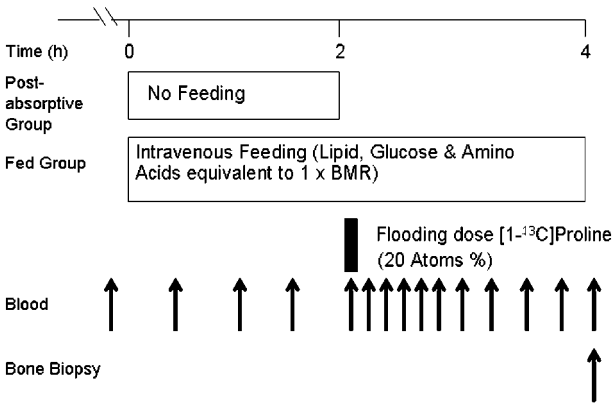

FIG. 1. Study protocols.

Because no previous data on human bone collagen synthetic rates existed, we assumed that the population SD was likely to be of the order of 15% (as we find routinely for human skeletal muscle) and that any increase after feeding was likely to be of the order of at least 25% and possibly up to 3-fold (as we routinely find for muscle protein synthesis).[13,14] Thus, setting probability at 0.05 and power at 0.85, and assuming no alteration of variance on feeding, using standard procedures (http://calculators.stat.ucla.edu/powercalc/), we calculated that, if our assumptions were correct, we should be able to comfortably detect an effect of feeding in two groups of eight subjects. We therefore studied one group of eight healthy men in the postabsorptive state and a separate group of eight healthy men in the fed state (mean values: age, 29 ± 3.5 [SD] years; BMI, 24.3 ± 2.1 kg/m^2; with no differences between groups). Subjects were randomly assigned to either the postabsorptive or fed group. Each subject brought with them a 24-h urine collection, taken immediately before the study. The subjects gave their informed consent, and the studies were approved by the Tayside Ethics Committee under the guidelines of the Declaration of Helsinki.

Protocols

Studies were carried out as shown in Fig. 1.

Basal postabsorptive group (n = 8): After an overnight fast, cannulae were placed in the antecubital veins of both arms to infuse stable isotope-labeled proline and, from the contralateral arm, to sample blood. In some subjects in these studies (4/16) and in related studies not reported here of tendon and muscle collagen synthesis, under local anesthesia (1% lidocaine) a small biopsy of skin (~10 mg) was taken using surgical scissors, and a quadriceps muscle biopsy was taken using the Bergström technique to obtain basal values of collagen hydroxyproline background labeling. A basal blood sample was taken, and a flooding dose of [1-^{13}C] (five subjects) or [^{15}N] proline (in three of the eight subjects; 20 Atoms %, i.e., 0.75 g of 99 Atoms % labeled; Cambridge Isotope Laboratories; and 3 g unlabeled proline) was given over 3 minutes. Blood samples were taken at 10- to 20-minutes intervals over 120 minutes, whereupon the subjects were lightly sedated (~5 mg midazolam), and bone biopsies were taken under local anesthesia of the superficial tissues overlying the hip and the periosteum (1%

lidocaine). Approximately 150 mg of bone was taken from the iliac crest using a spinal biopsy set (Bignall Surgical, Ford, Arundel, UK). Samples were frozen immediately in liquid nitrogen and stored at –80°C until analysis.

Fed group (n = 8): After an overnight fast, cannulae were placed into the antecubital veins to sample blood, administer stable isotope-labeled proline, and infuse 20% glucose (55% of energy), lipid (30% of energy; 20% Intralipid; Kabi-Fresenius), and a complete mixture of amino acids (15% of energy; Glamin; Kabi-Fresenius) equivalent to 100% of the subjects' basal metabolic rate, calculated using the Harris-Benedict equation.[15] Blood was taken before feeding and at 30-minute intervals thereafter for 120 minutes, whereupon a flooding dose of [1-^{13}C] proline (as above) was given over 3 minutes. The remainder of the procedure was identical to that described above.

Studies of possible interference of proline with protein synthesis in general

In developing the methods to be used in these studies, we wished to discover if there were general interfering effects of a large dose of proline on tissue protein synthesis assayed by the incorporation of other stable isotope-labeled amino acid tracers. To this end, we "piggy backed" flooding doses of proline onto separate ongoing studies of postabsorptive muscle protein synthesis carried out for other reasons and measured muscle protein synthesis as the incorporation of ^{13}C leucine, ^{13}C alanine, or ^{13}C proline into mixed or myofibrillar protein. The techniques used in these studies are as described in previous publications.[13,14,16]

Extraction and identification of procollagen and collagen

These methods have been described in detail previously.[16] Briefly, protein was sequentially extracted from bone using (1) 0.15 M NaCl, 0.2 M EDTA, and 0.05 M Tris-HCl, pH 7.4; (2) 0.5 M acetic acid (HOAc); and (3) 0.5 M acetic acid plus pepsin (0.1 mg/ml; P7125; Sigma) and hot (90°C) water. Isolated collagen fractions were dissolved into running buffer loaded onto a 6% SDS-PAGE gel and electrophoresed at 90 V for ~90 minutes. The gel was blotted onto a polyvinylidene fluoride membrane and exposed to antibodies to type I collagen (Rockland, Gilbertsville, PA, USA) and procollagen (Santa Cruz Biotechnology, Santa Cruz, CA, USA), with detection by chemiluminescent second antibodies.

Bone protein and procollagen and collagen concentrations

Isolated collagen fractions were analyzed both for nitrogen (as an index of protein concentration) by combustion-isotope ratio mass spectrometry (IRMS; Europa Scientific 20–20) and hydroxyproline (as an index of collagen) by gas chromatography–mass spectrometry (GC-MS; Fisons MD-800). Samples for nitrogen analysis were transferred to tin capsules and frozen in liquid nitrogen, and the solvent was evaporated under vacuum, combusted, and reduced to N_2 before analysis by IRMS for total nitrogen using a standard curve of responses of total ion current to known nitrogen amounts. To samples for hydroxyproline analysis, an internal standard was added where the samples were acid hydrolyzed and purified by cation exchange chromatography before their derivatization as *N*-acetyl *n*-propyl (NAP) esters. GC-MS was used to determine hydroxyproline concentration using a standard curve of responses to samples of known hydroxyproline concentrations, and collagen content was calculated using the assumption that hydroxyproline accounts for ~13% of collagen.[17]

Measurement of collagen hydroxyproline labeling and collagen synthetic rate

Isolated collagen fractions were hydrolyzed at 110°C overnight to release the protein-bound amino and imino acids in a slurry of 0.05 M HCl:Dowex 50WX8–200 (Sigma, Poole, UK) and the liberated amino acids eluted by NH_4OH, before their derivatization as NAP esters for analysis by GS–combustion–isotope ratio MS (GC-C-IRMS; Finnigan DeltaPlus -XL). Fractional synthetic rates (FSR, %/h) were calculated by comparing the increase in incorporation of tracer over time into bone collagen hydroxyproline with the area under the plasma ^{13}C or ^{15}N proline labeling-time curve (determined by GC-MS as their *t*-BDMS derivatives). The measurement of incorporation of label into hydroxyproline conferred specificity on the method for assay of rates of collagen synthesis only because no other bone protein contains hydroxyproline. In most studies ($n = 12$), we took as background enrichment the basal [^{13}C] hydroxyproline values determined from three unlabeled human neck of femur samples obtained at surgery (^{13}C Δ‰ v Pee Dee Belemnite [PDB] limestone of –32.205 ± 0.194). However, in some studies ($n = 4$), we used collagen isolated from skin biopsies to provide a background enrichment. In separate studies, we found that basal values of the enrichment of hydroxyproline in human collagen with ^{13}C and ^{15}N (i.e., without tracer having been applied) are identical in skin, muscle, and tendon, thus allowing skin to be used as a surrogate index of the basal background labeling for other tissues for which basal biopsies are difficult to obtain. [^{13}C] hydroxyproline basal values are as follows: skin, –32.27 ± 0.18; mixed muscle protein, –32.249 ± 0.19; tendon, –32.27 ± 0.18. For the same subject, skin versus muscle is –32.005 and –32.009. [^{15}N] hydroxyproline values (Δ‰ versus air N_2) are as follows: skin, 383.93 ± 2.33; muscle, 396.36 ± 23.25. In a single subject, skin and muscle values were 380.91 and 380.39, respectively. The relative constancy of basal hydroxyproline labeling means that, for subjects not having been exposed to stable isotope-labeled proline before, any collagen-containing tissue is likely to provide a suitable basal value; even if an assumed value representative of the population at study is used, as we did for neck of femur samples, any error thereby introduced is likely to be insignificant. This obviates the need for more than one bone biopsy.

Plasma and urinary marker analysis

The samples of plasma taken initially and after 240 minutes of intravenous feeding were used for measurement of plasma markers. Plasma procollagen type I N-terminal peptide (PINP) was measured using a commercial radioimmu-

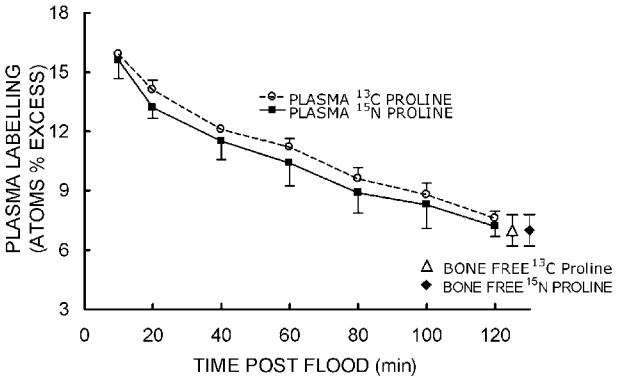

FIG. 2. Postabsorptive plasma ^{13}C and ^{15}N proline disappearance curves (○ and ♦) and bone free proline labeling (△ and ♦) in bone biopsies taken at 120 minutes. Values are means ± SD for five subjects for ^{13}C and three subjects for ^{15}N label.

noassay (Orion Diagnostica, Espoo, Finland), and osteocalcin was measured with an ELISA kit (Quidel Corp., San Diego, CA, USA). Urinary deoxypyridinoline and creatinine were measured in 24-h urine collections by ELISA kits (Quidel Corp.). All kits were used according to the manufacturers' instructions.

Statistical analysis

All data are expressed as means and SD. The variances of the two groups studied (postabsorptive and fed) were equal according to the *F* test, and therefore, unpaired Student's *t*-tests have been used to determine statistical significance as appropriate.

RESULTS

Validation of the assumptions underlying the method

Precursor enrichment: After injection of a large dose of proline, labeled with either ^{15}N or ^{13}C, the plasma proline concentration and labeling fell by ~50% over 2 h (Fig. 2), presumably because of dilution from protein breakdown, disappearance of proline into protein, and catabolism, in a variety of tissues, to glutamate.[18] When the values of labeling of free proline in the tissue water of human skin, muscle, and bone were measured at 2 h, they were within 6 ± 4% of the value obtained at time of the last plasma sample. No significant differences in labeling were discovered between any free proline pools, thus helping to validate the assumption that an equilibration of tracer had been achieved between accessible pools. Given that the labeling is similar in these pools, it is likely that the prolyl *t*-RNA is also labeled to the same extent. In pigs, we previously showed that the time course of labeling of free proline in dermis, bone, and plasma were very close[16] and contend that this was also likely in the studies of the subjects described here.

Flooding dose—lack of effect on protein synthesis: In humans, the application of large flooding doses of nonessential amino acids (which proline is) does not stimulate muscle protein synthesis[19]; more specifically, we have been unable to detect any effect of a flooding dose of proline on incorporation of alanine, delivered by constant infusion, into mixed muscle protein (synthetic rate without proline, 0.042%/h; with proline, 0.044%/h), and values of myofibrillar protein synthesis are very close when measured either with the proline flooding dose or with constantly infused [^{13}C] leucine (0.037 ± 0.007 versus 0.038 ± 0.009%/h, respectively; unpublished data).

The results show that we met the following requirements of the flooding dose approach: (1) equilibration of the label between the accessible, plasma proline pool, and the relatively inaccessible, intracellular pool and (2) the lack of interference of the flooding agent on protein synthesis.

Identical values were obtained whether ^{15}N or ^{13}C proline were used to make the measurement (Table 1). This widens the use of the method in studies of the effects of interventions, because successive measurements (e.g., before and after an intervention) are unlikely to interfere with one another because of the different isotope used and likely to accurately report the physiological state of the tissue.

In bone samples obtained by iliac crest biopsy from young men, we found that the isolated collagen fractions that had the lowest concentrations in bone of nitrogen (i.e., protein) and hydroxyproline (i.e., collagen) had the fastest rates of incorporation of ^{13}C or ^{15}N proline into hydroxyproline (Table 1). When isolated collagen fractions were subjected to SDS-PAGE and Western blotting and probed with antibodies against type 1 collagen and procollagen (Fig. 3), procollagen was found only in the NaCl-EDTA fraction. Progressively greater amounts of collagen were seen in the 0.5 M acetic acid, pepsin-acetic acid, and hot water extracts. We can tentatively assign identities to the four isolated fractions of collagen as (1) procollagen recently secreted by osteoblasts into the extracellular space, (2) tropocollagen (with some cross-linking), (3) highly cross-linked, more mature collagen, and (4) extensively cross-linked highly mature collagen. The ability to measure the rate of synthesis of collagen in these various pools is likely to be a useful attribute of the method in distinguishing between difficult models of collagen behavior in response to various physiological and pathophysiological stimuli.

Rates of collagen synthesis in human bone in the postabsorptive state

When we applied our technique to the measurement of iliac crest bone collagen synthesis in young healthy men, the values we obtained were much higher than expected (Fig. 4). The rate of incorporation of labeled proline into hydroxyproline was highest in the procollagen-containing fraction and lowest in the hot water extractable fraction; the average weighted rate (0.07%/h) of collagen synthesis (and thus in the steady state when synthesis equals breakdown, turnover) in the three main fractions was of the same order as that of mixed skeletal muscle protein (i.e., 0.05%/h[19]).

Effects of feeding on directly measured bone collagen synthesis

We have previously shown that skeletal muscle protein synthesis is acutely sensitive to stimulation by amino acids in a dose-dependent fashion within one-half an hour of their increased availability, the stimulation lasting about 2.5

Table 1. Bone Protein and Collagen Concentrations and Collagen FSR

Extract	Protein (μg/mg bone wet weight) (n = 6)	Collagen (μg/mg bone wet weight) (n = 6)	Postabosrptive collagen FSR (% h)		Fed collagen FSR (% h)
			^{15}N proline (n = 3)	^{13}C proline (n = 5)	^{13}C proline (n = 8)
Acetic acid	12 ± 2	4 ± 0.5	0.21 ± 0.04	0.24 ± 0.05	0.44 ± 0.05*
Acetic acid/pepsin	29 ± 8	13 ± 3.2	0.07 ± 0.01	0.07 ± 0.02	0.11 ± 0.01*
Hot water	168 ± 28	128 ± 18	0.06 ± 0.01	0.06 ± 0.01	0.11 ± 0.01*

Values are means ± SD.
* $p < 0.01$ between postabsorptive and fed, unpaired Student's *t*-test.

h.[14,20] However, we had no a priori view of the relative importance of particular nutrients or hormones for bone. We therefore studied the response of bone collagen in young healthy men during the provision of a mixture of triglycerides, glucose, and amino acids delivered intravenously. The results (Fig. 4) show a marked stimulation of collagen synthesis in the isolated HOAc, Pepsin-HOAc, and hot water fractions of bone collagen synthesis in the second 2 of 4 h of intravenous feeding. The stimulation occurred entirely in the acetic acid, acetic acid plus pepsin, and the hot water extractable fractions. We were unable to detect any labeling in the NaCl-EDTA fraction, which seemed to contain an immeasurably small amount of procollagen. When calculated as a mean weighted rate, the increase was ~60%, a value likely to have been observed in whole mixed bone collagen.

Effect of feeding on plasma markers of bone formation

Although we saw a rapid and marked increase in directly measured bone collagen synthesis in response to intravenous feeding, plasma PINP and osteocalcin (indirect markers of collagen synthesis and osteoblast differentiation/bone mineralization, respectively) remained at the postabsorptive values (Table 2). We did not attempt to measure the effect of feeding on urinary deoxypyridinoline excretion, but the average rate in our subjects in the 24-h period before feeding was 4.3 ± 1.0 nmol/mmol creatinine, which is in agreement with published rates of production for a healthy adult (4.7 ± 1.6 nmol/mmol creatinine).[21]

DISCUSSION

Our results provide new insights into human bone collagen metabolism: they suggest that, in the iliac crest at least, its synthesis (and hence its turnover) is faster (~1.6%/day) than expected, given generally accepted values of adult cortical and trabecular bone remodeling, respectively, of 3–25% per year[22] or of bone calcium turnover of ~8–15% per year.[23,24] The discrepancy in values may be caused because of errors in our measurements, because the values for iliac crest collagen synthesis are unrepresentative of bone generally, or because the prevailing paradigm is in error and values obtained from bone histomorphometry and bone markers are unrepresentative of bone collagen turnover for reasons not yet understood.

How robust are our results and how may they be placed in context? In methodological terms, it seems unlikely that the fundamental measurements we made of the extent of labeling in bone collagen hydroxyproline and plasma proline, which are the basis of our calculations of collagen synthetic rate, are incorrect. The GC-MS measurements are robust, and using almost identical techniques, we obtained values for protein synthetic rates (e.g., human muscle and gastrointestinal), which are similar to those of other workers[25–27] and are widely accepted among the physiological community.

However, one possibility is that proline used in the amounts we gave in the flooding dose stimulates bone collagen synthesis. We find this hard to accept because this has never been previously reported for any system, we know from our own data that proline does not stimulate mixed muscle protein synthesis, and furthermore, the values we obtain for human muscle (mainly perimysial) collagen synthesis measured using the same techniques bear the same relationship to mixed muscle protein synthesis (a ratio of ~1:4) as reported in rat muscle.[28] In addition, in circumstances in which a flooding dose of essential amino acids, particularly leucine, does seem to stimulate tissue protein synthesis,[29] it is difficult to further stimulate the system by feeding,[30] which we are able to do in both muscle and bone (see below).

Another problem is the difficulty of unrepresentative sampling. We took samples from iliac crest and made measurements on fractions of collagen extracted by differential solubility in various reagents. Could differences between regions of bone of different metabolic characteristics explain the possible discrepancy between conventional turnover rates and those we found? It is possible that iliac crest is unrepresentative, but we know of no data mapping the amounts of bone of different characteristics or quantitative estimates of their contributions to the whole body; therefore, we are unable to provide a definitive estimate of any possible error, but we think it unlikely to be as much as 10-fold, the margin between collagen synthesis rates and other estimates of bone collagen turnover. Any differences attributable to the type of bone sampled seems unlikely to us because we have, in separate studies, measured rates of collagen synthesis in cortical, trabecular, and corticotrabecular bone obtained from patients undergoing hip replacement for osteoarthritis, and the range of values between bone of different types was no more than 30%.[31]

How do the current results compare with values of collagen turnover inferred from indirect measurements of collagen peptides and excreted cross-linkers? This is a difficult question to answer because the assay of procollagen peptides in tissue fluid, plasma, or serum provides static mea-

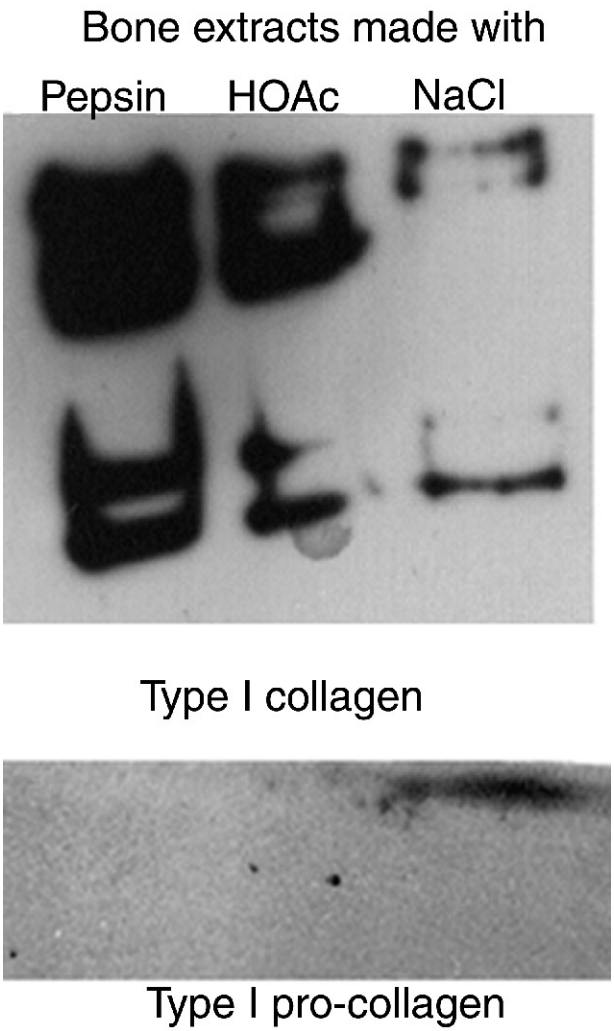

FIG. 3. Western blots of isolated bone collagen fractions from postabsorptive subject using specific type 1 collagen (top) or type 1 procollagen (bottom) antibodies. HOAc, acetic acid.

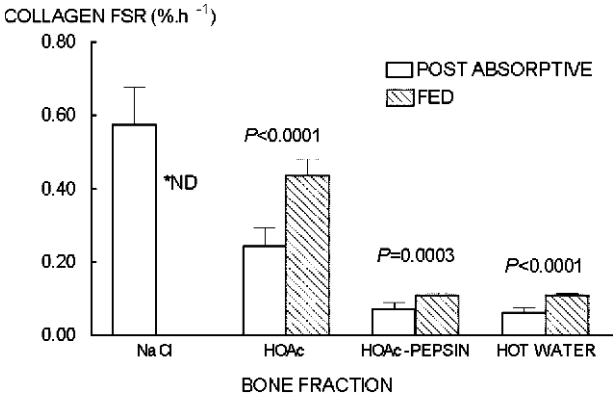

FIG. 4. Comparison of postabsorptive and feeding collagen fractional synthetic rates (HOAc = acetic acid). Values are means ± SD for eight subjects per group. *ND, not detectable by GC-C-IRMS; $p < 0.01$, probability of significant difference between postabsorptive and fed states, unpaired Student's *t*-test.

TABLE 2. DIRECT AND INDIRECT MEASURES OF BONE FORMATION PRE- AND POSTFEEDING

Measured variable	*Fasted*	*Fed*
Mature bone collagen FSR (% h)	0.06 ± 0.01	0.11 ± 0.01*
Plasma PINP (μg/liter)	54.9 ± 21.3	44.7 ± 14.6
Plasma osteocalcin (ng/ml)	10.7 ± 1.8	9.2 ± 2.9
Urinary DPD (nmol/mmol creatinine)	4.3 ± 1.0	—

Means ± SD.
* $p < 0.01$ unpaired Student's *t*-test.

sures, which give no kinetic information. In separate studies,[32] we compared the time course of exercise stimulation of patella tendon collagen synthesis measured directly by our new method with the appearance of PINP in peritendinous fluid sampled by tissue dialysis, with large pore membranes (~3000-kDa cut-off). We found that the response of PINP was sluggish and attenuated, taking at least 24 h to rise to a peak lower than that observed in directly measured tendon synthesis rate at 6 h. In this study, we saw no acute response of plasma PINP or osteocalcin to feeding within 2 h, despite the large stimulation of collagen synthesis observed when measured directly. Thus, we conclude that PINP and osteocalcin are uncertain indices of acute changes in bone anabolism.

What light do the findings throw on the relationship between collagen synthesis and breakdown? In the steady state, these processes must be equal and opposite. However, the rates of urinary excretion of the breakdown markers N- and C-terminal telopeptides NTx and CTx), pyridinoline, and deoxypyridinoline (DPD) are difficult to interpret because they come from collagens of different types originating in different tissues. Although it is often asserted that the type 1 collagen in bone is the major contributor to DPD in urine, its concentration in bone is only ~3-fold greater than that in skeletal muscle (16 versus 5 nmol/g dry tissue),[33] and there is about twice as much skeletal muscle as bone (28% versus 14%) in the body.[34] Thus, the urinary concentration of DPD may be biased by skeletal muscle collagen breakdown.

How does the directly measured rate of collagen synthesis relate to turnover of bone from racemization/isomerization of CTx and DPD measures? There are many discrepancies. For example, the results of Gineyts et al.,[33] based on the degree of racemization of collagen residues, gives an order of collagen turnover as muscle > tendon = ligament > bone > dermis. Our data based on direct tracer incorporation measures give the order as bone > dermis > tendon = ligament > muscle. Furthermore, because pool size × synthetic rate = absolute rate of synthesis and synthesis ≃ breakdown in steady state, muscle DPD (nmol) × muscle collagen fractional synthetic rate (%/day) + bone DPD (nmol) × bone FSR (%/day) ≃ whole body DPD production.

Taking the average muscle mass as 28 kg and the average bone mass as 14 kg, and ignoring the effects of feeding on FSR, regional differences in bone composition, and any processing of DPD before excretion, we obtain:

(3 nmol/g × 2800 g × 0.42%/day) + (17 nmol/g × 14,000 × 1.7%/day) = 4000 nmol/day

However, measured urine excretion rates are only ~100 nmol/day (i.e., there is a 40-fold discrepancy. Similar calculations can be made using the data of Gineyts et al.[33] for urinary NTX, with similar results. It is impossible, a priori, to decide which approach is correct. However, if our values prove to be correct, this means that the measurable amounts of collagen markers in biological fluids must have been the result of considerable processing and destruction during their journey to plasma or urine.

There seems to be no obvious way to resolve this conundrum except by further research (e.g., by applying, simultaneously and separately, the flooding dose technique using say [^{13}C] proline and a constant infusion technique with [^{15}N] proline in an animal large enough to obtain bone prolyl *t*- RNA in sufficient amounts to provide a definitive answer).

The observation of marked increases in the synthesis of bone collagen in response to feeding is an exciting result, especially given the rapidity and size of the response, but it was predictable: in osteoblasts in tissue culture, and in other animal preparations, it is possible to stimulate collagen synthesis by amino acids and IGF-I.[35–39] Bone collagen mass decreases in animals with fasting[40] and may be protected from wasting in animals and human beings by feeding milk basic protein.[41,42] Furthermore, in the osteoporotic elderly, risk of hip fracture is greater in those with a habitually low-protein diet,[43] whereas protein supplementation raises serum IGF-I and attenuates bone loss in patients with recent hip fracture.[44]

We do not yet know with certainty which of the nutritional components stimulated bone collagen synthesis in our subjects who received glucose, fat, and amino acids, but we predict from our work on muscle[14,45] that amino acids are the most likely candidate. We have shown in separate studies that muscle protein synthesis may be acutely modulated in a dose-response manner by both mixed and essential amino acids,[14,20] and we predict that the same will be true of bone collagen synthesis so that the protein content of the diet may have a direct effect on bone maintenance. Nevertheless, unsaturated fatty acids may also have a nutritionally important role through the stimulation of growth factors such as TGF-β1, which regulates lysyl oxidase activity,[46] and through the increased availability of n-3 unsaturated fatty acids, which reportedly stimulate bone formation and prevent bone loss in animals.[47]

What are the implications of our results for human health and welfare and for the development of successful approaches to the treatment of bone disorders? First, good descriptions of exactly what happens to human bone collagen synthesis during development and in health and disease are lacking. This gap could now be filled. Second, treating bone wasting using pharmaceutical approaches has not proved particularly successful, and although the most widely used drugs, the bisphosphonates, do decrease fracture rates, they do little to improve bone quality.[6] Because of the health risks associated with its use, hormone replacement therapy is no longer recommended as a treatment for osteopenia and osteoporosis in women,[48] and there is little unequivocal evidence that alternative treatments (such as use of vitamin D and calcium in postmenopausal women) are of substantial benefit. This means that the search for new means of ameliorating bone wasting is important. The fact that bone collagen synthesis is apparently so rapid and may be measured repeatedly by the use of different isotopically labeled tracers (e.g., ^{15}N and ^{13}C proline and possibly $^{18}O_2$ to label bone hydroxyproline) means that new therapeutic candidates (whether lifestyle changes or pharmaceutical agents) that increase bone formation could be tested quickly, within a matter of days to weeks in the same subjects.

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