0163-769X/96/$03.00/0
Endocrine Reviews
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Vol. 17, No. 4
Printed in U.S.A.

Molecular Basis and Clinical Application of Biological Markers of Bone Turnover*

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I. Introduction

THERE is increasing awareness among scientists, clinicians, policy makers, and the general public of the costs and health care problems associated with osteoporosis, the most common metabolic bone disease. If the disease could be prevented or effectively treated, then deaths, disabilities, and costs due to osteoporosis would be substantially reduced. To this end, considerable emphasis has been placed on developing and improving indicators of bone remodeling for 1) identifying people at risk, 2) early diagnosis, and 3) determining effective therapy for those with established disease. Although the clinician's ability to diagnose and monitor bone disease has improved in the past decade, there is still a need for more specific methods of assessing disturbances in bone metabolism.

Bone status can be assessed by dynamic histomorphometry of a biopsy specimen, but the technique is invasive, and results from a single core biopsy may not apply to other sites in the skeleton. Quantitative bone absorptiometry offers an accurate assessment of bone mass, but only of structural changes that occur over several years in the individual patient. Direct serum measurements are useful because they allow for easy and frequent assessment without undue risk or discomfort. However, the classic biochemical markers of bone disease, serum total alkaline phosphatase activity and urinary total hydroxyproline excretion, are strongly influenced by nonosseous metabolism and are subject to interference from systemic disorders. Furthermore, the changes observed are often modest. Therefore, there is a compelling need for new and/or improved biochemical markers that would accurately assess dynamic changes in bone remodeling.

Here we will review recent progress in refining and improving diagnostic markers of bone metabolism and their status in clinical application. The aim is not to provide comprehensive review of clinical studies involving old and new markers. The literature is too vast and increasing rapidly. Rather, we will describe the biochemical basis and development of each marker and compare different assay methods. Clinical findings will be summarized with an emphasis on areas of controversy and specific needs for further research and assay development. After the various markers are described individually, comparative studies that evaluate the relative clinical usefulness of the various markers are reviewed. Finally, *from our perspective*, we make suggestions on the current and potential use of bone markers. We begin with a brief summary of bone biology and collagen biochemistry to orient the reader, since four of the seven markers described are based on collagen metabolism.

A. Overview of the basic biology and biochemistry of bone

1. Biology. The skeleton provides the mechanical support of the body and a reservoir for normal mineral metabolism. In both capacities, bone is an active tissue constantly being remodeled and changing metabolically. The cells that mediate change, osteoblasts and osteoclasts, have been extensively studied. The resident cells, osteocytes, are less well understood. Although cells account for only a small fraction of bone volume, their function is essential. In concert with calciotropic hormones, they regulate the balance of mineral

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* Supported by the Food and Drug Administration and by NIH grants RO1-AR-38460 (to C.M.G.) and RO1-AR-37318 (to D.R.E.). Presented in part by Drs. Calvo and Gundberg to the Office of Technology Assessment as part of a report to the Congress of the United States on "Current Status and Future Needs for Osteoporosis Research, Education, and Medical Screening 1990–1993."

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between bone and blood, keeping serum calcium and phosphorus in narrow concentration ranges (1).

Bone cells also participate in the growth, modeling, and remodeling of bone. The two major types of bone are trabecular or cancellous bone, a bony lattice that has a spongy appearance, and cortical bone, a layered solid structure. In addition to these structural differences, trabecular and cortical bone differ in the spatial arrangement of their cells, in the density of the mineralized matrix, and in the distribution of blood vessels and marrow that bathe and nurture the bone cells. For both types of bone, osteoblasts and osteoclasts move freely along the surface, and the osteoblasts may ultimately become embedded in the mineralized tissue as an osteocyte. Osteocytes remain in limited contact with the blood supply and extracellular fluids. Trabecular bone has a larger surface area and is more metabolically active than cortical bone (2).

During childhood and adolescence growth of the skeleton involves accumulation of skeletal mass. Bone growth and modeling ends with epiphyseal closure, but additional mineral is deposited during a period of consolidation. Throughout life the skeleton undergoes continuous remodeling (turnover) of bone with removal of old bone and replacement with new bone. This allows the skeletal system to respond to outside mechanical forces or molecular signals. In the normal adult skeleton, this process takes place at discrete sites (remodeling units) and over a fixed period of time (usually about 90 days)(3). Bone turnover is always initiated by osteoclasts eroding a mineralized surface. This process is followed by the recruitment of successive teams of osteoblasts to the outer edge of the erosion cavity that secrete new bone matrix (osteoid) and gradually fill in the resorption cavity (4).

Both systemic and local factors influence bone growth and turnover. PTH and the active form of vitamin D, 1,25-$(OH)_2D_3$ (calcitriol), stimulate bone resorption, while calcitonin inhibits it (5). Bone resorption by osteoclasts and bone formation by osteoblasts are also regulated by a variety of cytokines and growth factors. In the steady state, this "coupling" of bone formation and resorption maintains bone mass. After growth ceases, any unbalance can lead to debilitating bone disease. Resorption that exceeds formation, for example, after the menopause, results in osteoporosis.

2. *Biochemistry*. Osteoid matrix consists principally of collagen (90%), other smaller matrix proteins, and proteoglycans. It is rapidly mineralized in close apposition to and throughout the collagen fibrils. The main structural protein of bone is type I collagen. By definition, all collagens contain molecular domains of triple-helical conformation that require the repeating sequence (*Glycine-X-Y*) where X and Y are most often proline and hydroxyproline, respectively. Three α-chains fold to form the triple helix. Type I contains two $\alpha 1$ (I) chains and one $\alpha 2$ (I) chain (6). Figure 1 summarizes the intracellular events in type I collagen synthesis. The newly translated polypeptide, a pre-pro-α-chain, includes a signal sequence and amino (N)- and carboxyl (C)-terminal propeptide extensions (7). Before translation is complete, it appears that most of the prolyl and lysyl residues on the nascent α-chains are hydroxylated. Before the triple helix folds, certain hydroxylysyl residues are glycosylated to galactosylhydroxylysyl or glucosyl-galactosyl-hydroxlysyl residues (8). In type I collagen, intramolecular disulfide bonds form between the three carboxyl propeptides. These extension domains may guide triple helical formation. The procollagen molecule is then transported from the rough endoplasmic reticulum to the Golgi for additional glycosylation and sub-

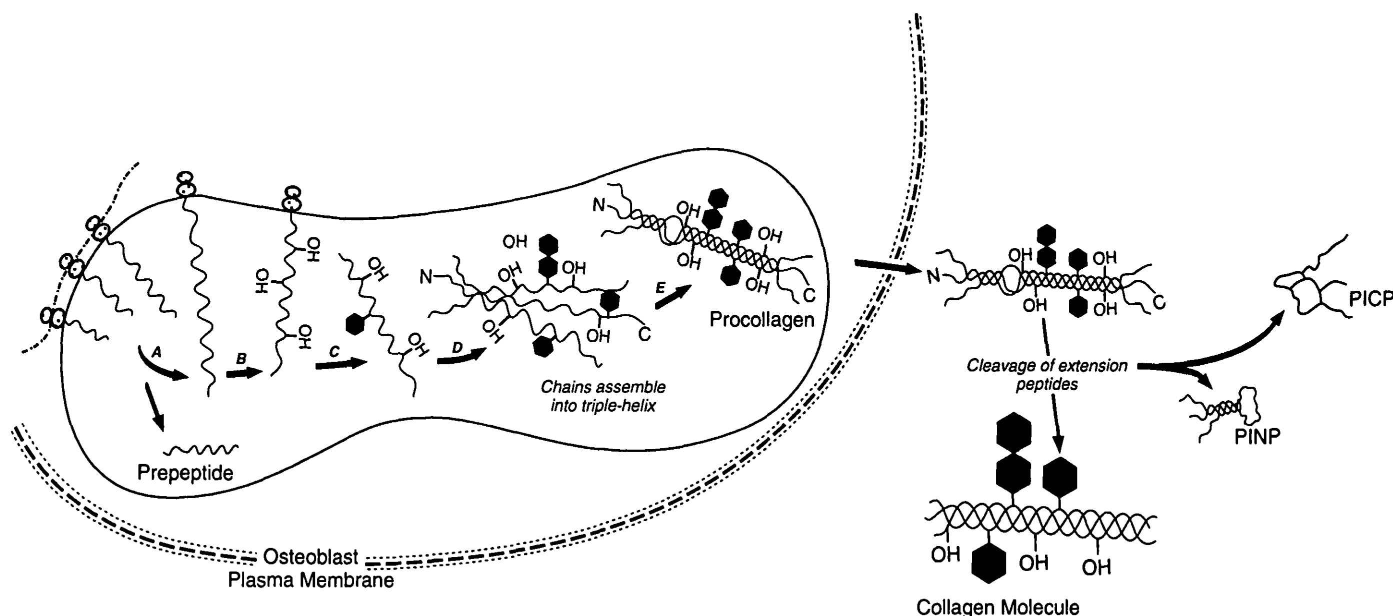

FIG. 1. The intracellular pathway of type I collagen synthesis. A, Type I collagen polypeptides are synthesized as pre-pro-α-chains that contain a peptide-secretory sequence and amino (N)- and carboxyl (C)- terminal extension peptides. B, Many of the prolyl and lysyl residues on the nascent α-chains are hydroxylated before translation is complete. C, The resulting hydroxylysyl residues are glycosylated to form GHYL or Glc.GHYL residues. D, Three pro-α-chains are associated into a triple helical molecule that is stabilized by disulfide bonds between the carboxyl propeptides. E, These extension peptides guide helical folding of the molecule which, once this has occurred, is transported to the Golgi for subsequent secretion. After secretion, the N- and C-terminal extension peptides are cleaved by specific peptidases, and the extension peptides can enter the circulation. A fraction of the N-terminal peptides, however, may bind to bone matrix.

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sequent secretion into the extracellular space (9). Once secreted, and during fibrillogenesis and fibril maturation, the N- and C-terminal propeptides are cleaved by specific peptidases, and these can enter the circulation. In bone, the N-propeptide of the α1 (1) chain has been found in extracts of the mineralized matrix.

Collagen molecules aggregate in a staggered array to form fibrils that are strengthened by covalent cross-links. These are catalyzed by the action of lysyl oxidase, which forms aldehydes from certain lysine and hydroxylysine side chains. The condensation of lysyl and hydroxylysyl residues in adjacent molecules results in various types of cross-linking residues. When the aldehydes are derived from hydroxylysine, the mature cross-links are 3-hydroxypyridinium structures called pyridinolines (10)(Fig. 2).

When osteoclasts resorb bone, they secrete a mixture of acid and neutral proteases that act sequentially to degrade the collagen fibrils into molecular fragments. The precise extent of degradation by the osteoclast alone is unknown. Circulating products containing the pyridinoline cross-links range in size from the free amino acids to segments of the N-telopeptide and C-telopeptide domains (10). Presumably the initial fragments produced by osteoclasts are further metabolized by the liver and kidney so that eventually all the cross-link-containing fragments are of sufficiently small molecular weight to be cleared by the kidneys and excreted in the urine. In summary, circulating peptides of type I collagen can arise from two sources: osteoblastic synthesis of bone matrix (N- and C-propeptides) and degradation products from osteoclastic activity (cross-linked N- and C-telopeptides).

Other markers of bone turnover are not related to collagen synthesis or catabolism. Osteocalcin is a small protein synthesized by osteoblasts that becomes part of the bone matrix. Some of it spills over into the circulation where its concentration is a commonly used marker of bone formation. Markers of bone metabolism also include enzyme levels in serum that arise from osteoblast or osteoclast activity. Examples include bone-specific alkaline phosphatase, a formative marker, and tartrate-resistant acid phosphatase, a resorptive marker.

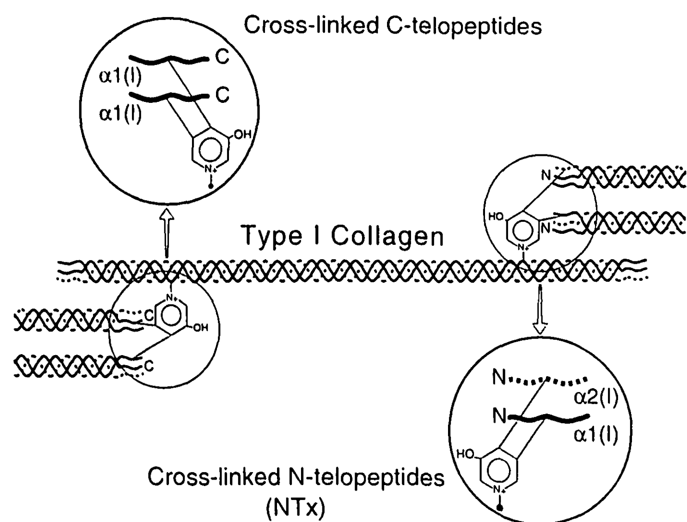

FIG. 2. Cross-linked N- and C-telopeptides of type I collagen. The pyridinoline cross-links occur essentially at two intermolecular sites in the collagen fibril: two aminotelopeptides are linked to a helical site at or near residue 930 (NTx), and two carboxytelopeptides are linked to helical residue 87 (CTx).

B. Criteria for ideal markers of bone turnover

The most critical characteristic of an effective resorption or formation test is that it is minimally invasive. Blood sampling and urine collection allow for easy and frequent measurement over time without undue risk or discomfort to the patient. Ideally, the analyte should be unique to bone, or more specifically to the osteoblastic formation process or the osteoclastic resorption process. To be validated, the marker should 1) correlate with a standard reference of bone-remodeling activity such as stable isotopes, radio-calcium kinetics (^{47}Ca), and bone histomorphometry; 2) correlate to measured changes in bone mass; and 3) respond appropriately after treatment in diseases known to affect bone formation or resorption. Unlike localized measurements of bone turnover that reflect activity at a specific site, systemic biochemical markers will reflect collective remodeling throughout the entire skeleton and may not be confined to cortical or trabecular bone. Factors that affect the marker's levels, including circadian rhythmicity, diet, age, gender, body and bone mass differences, physical and metabolic activity, renal function, comorbid conditions, and drugs, should be clearly defined and appropriately adjusted whenever possible.

Most of the traditional and new markers for bone resorption measure the collagen degradation products from osteoclast activity and include urinary hydroxyproline, hydroxylysine glycosides, total or free pyridinoline cross-links, and cross-linked N- or C-telopeptides. Only one marker, serum tartrate resistant-acid phosphatase, is based on an enzyme secreted by osteoclasts. The formation markers are all osteoblast products that enter the circulation. These include serum alkaline phosphatase, osteocalcin, and type I procollagen peptides. Fasting urinary calcium, used by some investigators to measure bone resorption, is not discussed, and readers are referred to other works for discussion of this parameter (11, 12).

II. Indices of Bone Resorption

A. Hydroxyproline

1. Biochemistry. Urinary hydroxyproline, the most performed measure of bone resorption, has the longest history of use. Both hydroxyproline and hydroxylysine are amino acids essentially unique to collagenous proteins. Bone may be the primary store of collagen in the body, but both hydroxyproline and hydroxylysine are present in essentially all tissues and all genetic types of collagen. The majority of hydroxyproline derived from the breakdown of collagen is reabsorbed by the renal tubules and broken down in the liver. Only about 10% of hydroxyproline-containing products from collagen breakdown are excreted in the urine. Of that, most is contained in peptides, the majority of which are di- and tripeptides (13–15). The remaining peptides in the urine are of approximately 5 kDa and may be derived from the

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N-terminal extension peptide of procollagen (N-propeptide). There is a small amount of the free imino acid in urine.

Hydroxyproline can never be reincorporated into newly synthesized collagen (16). However, both collagen synthesis and tissue breakdown contribute to urinary hydroxyproline. Early studies using [^{14}C]proline showed that one-third to one-half of the hydroxyproline excreted by young rats originated from the catabolism of newly synthesized collagen that was not incorporated into tissue. In contrast, most of the hydroxyproline excreted by older rats came from structural collagen breakdown (16, 17). The small pool of urinary hydroxyproline peptides thought to originate from the N-propeptide of type I collagen was proposed as a marker of bone formation, but this has not been pursued (18–21).

In addition to all structural collagens, hydroxyproline also occurs in certain serum proteins, the most noteworthy of which is a component of complement, C1q (22, 23). As part of a minor serum protein, its contribution to the total load of hydroxyproline excreted in the urine is small compared with dietary sources. Normal ingestion of gelatin or collagen-rich foods such as meat can increase the level of urinary hydroxyproline (24). The urinary peptides containing hydroxyproline from endogenous collagen breakdown are indistinguishable from the dietary peptides (21). Consequently, to use urinary hydroxyproline most effectively, some experts suggest restricting intake of gelatin and other collagen-rich foods for 24–48 h before a 24-h urine collection (25). Table 1 lists the factors that contribute to urinary hydroxyproline.

Urinary hydroxyproline excretion shows a circadian rhythm with peak excretion after midnight; therefore, collection times need to be standardized if 24-h urine collections are not used (26–28). Also, Mautalen and Casco (28) noted a change in the the hydroxyproline-creatinine ratio over 24 h. They and others suggested using spot urines taken at the same time of day or expressing the hydroxyproline content of 24-h samples per square meter of body surface for meaningful comparisons (28, 29). Currently the accepted collection method for hydroxyproline analysis is 1- to 2-h "spot" urines made after a 10- to 12-h fast (26, 27).

2. Methods. Many methods for the assay of hydroxyproline in urine have been published. Discussion here focuses on the simpler methods in clinical use (30–41). Despite considerable improvements in precision, the assays are still tedious and time consuming. Since 90% of urinary hydroxyproline is peptide-bound, an initial hydrolysis step is required. Colorimetric techniques are based on the oxidation of the amino acid to pyrrole 2-carboxylic acid, which is converted to pyrrole upon heating (30–37). The pyrrole is then extracted (usually with toluene) and reacted with *p*-dimethylaminobenzaldehyde (Ehrlich's reagent) to form a chromophore that is quantified spectrophotometrically. In these colorimetric assays, interfering chromophores are the main source of error. Although conveniently adapted to the autoanalyzer, the method remains tedious due to the acid hydrolysis step (33, 36). A modified colorimetric assay is available as a commercial kit, Hypronosticon (Organon Teknika Inc, Scarborough, Ontario, Canada) (35). This procedure uses a patented resin tablet to remove interfering substances and hydrolyze peptides (40). Colorimetric methods report inter- and intraassay coefficients of variation of 10 and 12%, respectively, with detection limits of 5–10 μmol/liter (26, 37).

Newer HPLC methods for total urinary hydroxyproline derivatize the amino acid with phenylisothiocyanate and quantify the phenylthiohydantoin derivative by reversed phase-HPLC and UV absorbance. This method eliminates tedious sample clean-up and is easily adapted for use with an automatic sample processor for overnight assay. For the HPLC method, total coefficients of variation are 5.3% for a high pool and 3.9% for a low pool. The assay is of sufficient sensitivity such that only 50 μl of urine are necessary for samples to fall within the linear range (0–660 μmol/liter) The normal range for hydroxyproline excretion in men on unrestricted diets is 123–308 μmol/24 h. (38, 39, 41).

3. Clinical correlates. Despite its lack of tissue specificity, poor ability to differentiate between bone formation and degradation, and many confounding influences, urinary hydroxyproline has been one of the most widely used biological markers of bone turnover. In several studies in which histomorphometry or calcium kinetics have been used, hydroxyproline excretion correlated with calcium accretion and bone resorption in subjects with osteoporosis, Paget's disease, hyperparathyroidism, hyperthyroidism, and hypothyroidism (42, 43). Strong correlations between urinary hydroxyproline excretion and both mineralization and resorption rates (43) reflect the tight coupling between bone formation and bone resorption.

Age and gender influence hydroxyproline excretion (44,

TABLE 1. Factors contributing to variations in urinary hydroxyproline

Diet	All types of meat, fish, and poultry (highest sources: hot dogs, bologna, sausages, and poultry prepared with the skin on); gelatin, and gelatin added to products like yogurt; gelatin capsules used to administer fecal markers or test compounds or as coatings on medications.
Collagen synthesis	All connective tissues contribute (skin, tendon, fascia, bones, etc.) especially during growth.
Collagen degradation	All connective tissues contribute; can be elevated in chronic disease states without bone involvement such as arthritis and in weight loss.
Larger body size	Reflects greater connective tissue mass; requires normalization with creatinine.
Circadian rhythm	Highest excretion at night; requires complete 24 h or standardized times for incomplete urine collections.
Serum proteins	C1q component of complement contains hydroxyproline; others may exist, but noncollagenous sources are minor contributors.

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45). Gender differences in hydroxyproline excretion relate to body size and disappear when normalized to creatinine. The normal ranges for urinary hydroxyproline-creatinine ratios (mg/mg/24 h) for men and women on restricted diets are 0.019–0.032 and 0.022–0.036, respectively. Levels are highest in infants, with a mean of approximately 0.5 at 2–3 days of age to 2.0 at 33 days. Levels decline steadily until age 5 (0.38 at 6 months to 0.125 at 5 yr). Excretion remains stable until puberty, after which point it declines to the adult levels. No racial differences were noted between black and white children (46), but hydroxyproline-creatinine ratios were lower in healthy postmenopausal black women than in age-matched white women (12). In growing children, the higher levels probably reflect both bone collagen synthesis and resorption. In adults and the elderly, urinary hydroxyproline levels are influenced by total bone mass, body size, and renal function (26, 47–49). To correct for these influences, urinary hydroxyproline is often expressed in relation to bone mass, body weight, total surface area, 24-h creatinine excretion, or glomerular filtration rate.

Urinary hydroxyproline has been used effectively to diagnose and monitor the effects of therapeutic treatment of Paget's disease (50) and osteoporosis (51) and to monitor the short- and long-term changes in bone turnover caused by calcium supplementation in postmenopausal women (52–54) and normal men (55). Several studies report higher urinary hydroxyproline excretion in postmenopausal and amenorrheic women (56–62). Bone mineral content of the distal radius was inversely correlated to hydroxyproline-creatinine ratio in postmenopausal women (63). Hyldstrup and coworkers (47) demonstrated an increase in bone resorption per unit of bone in postmenopausal women after correcting hydroxyproline-creatinine ratio for bone mineral content. In women with surgically induced menopause (64), hydroxyproline excretion was strongly correlated to the rate of bone loss. Deacon *et al.* (65) validated the use of hydroxyproline as a marker of bone resorption in osteoporotic women by comparing its rate of excretion with kinetic estimations of bone resorption (^{85}Sr isotope tracer). Hydroxyproline excretion measured over 5 days was comparable to the isotopic method.

It can be argued that hydroxyproline excretion is not a sensitive enough marker of bone resorption for routine diagnostic and therapeutic monitoring in individual patients (57) because it lacks specificity and is easily confounded by diet. Nevertheless, in group studies, hydroxyproline excretion has been used recently to demonstrate increased bone resorption with immobilization (66), to monitor calcitonin treatment in postmenopausal women (51, 67–69), and to evaluate rate of bone resorption and formation in osteoporotic women during continuous or intermittent PTH therapy (70). In a study that used biochemical markers to evaluate intermittent PTH injections, urinary hydroxyproline corrected for glomerular filtration rate was the most consistent, and the only marker to increase progressively during PTH injection, to correlate with osteoclast number, and decrease with calcitonin therapy (70). However, it will be apparent from the following discussion of new bone resorption markers that hydroxyproline excretion does not perform as consistently as the newer resorptive markers. It is important to note, however, that in only a few of these clinical studies was urinary hydroxyproline collected under conditions that would limit confounding influences such as diet. Information is rarely given about the precision of the hydroxyproline assay, and interassay coefficient of variations (CVs) are reported as high as 20%. While hydroxyproline excretion as a marker of bone resorptive activity is clearly less than ideal, when measured correctly, it can provide useful information.

B. Galactosyl hydroxylysine (GHYL)

1. Biochemistry. Hydroxylysine, another modified amino acid peculiar to collagens, is glycosylated to varying degrees depending on the tissue type (71). Two glycosides are formed, GHYL and glucosyl galactosyl hydroxylysine (Glc.GHYL), which also appear in the urine (Fig. 3). While hydroxylysine and its glycosides are less abundant than hydroxyproline in bone collagen, certain properties make hydroxylysine theoretically a better marker of bone turnover than urinary hydroxyproline. Glycosylated hydroxylysine residues appear not to be reused or catabolized when collagen is degraded; therefore, urinary glycosylated hydroxylysines may represent the total pool of degraded collagen in the body. Furthermore, they do not appear to be absorbed in significant levels from a normal diet (71).

Because of tissue-specific differences in the ratios of GHYL and Glc.GHYL, GHYL is considered relatively specific to bone collagen degradation (72). Thus, about one third of the hydroxylysine residues in bone and skin collagens are glycosylated, but in humans Glc.GHYL predominates in skin, whereas GHYL is the main glycoside in bone (73). The ratio of Glc.GHYL/GHYL in skin is 1.6:1 whereas the ratio in bone is 1:7 (72, 73). The ratio of Glc.GHYL/GHYL in urine is

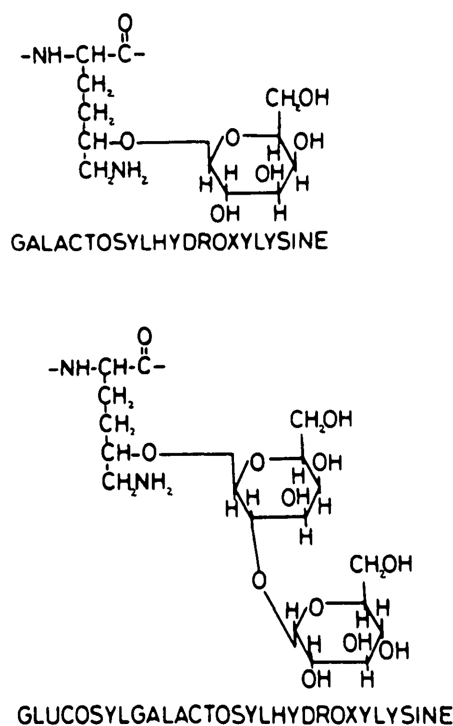

FIG. 3. Structure of hydroxlysine glycosides.

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therefore influenced by the predominant type of collagen being degraded and also by the age of the subject.

However, as with hydroxyproline, hydroxylysine glycosides are present in proteins other than structural collagens, *e.g.* the C1q component of complement. Their contribution to the urine pool is thought to be small. Clq contains only five residues each of hydroxyproline and hydroxylysine (22, 23), and most of the glycosylated hydroxylysine is Glc.GHYL. Another potential problem is degradation of the Glc.GHYL to GHYL (74). However, the α-glucosidase present in the rat renal cortex that can convert diglycosides to monoglycosides was shown to be absent from human kidneys (75). In humans, GHYL appears to be specific for bone collagen degradation, and both Glc.GHYL and GHYL seem to be the final metabolic products of collagen degradation.

In normal urine, 80% of the total hydroxylysine is in the form of hydroxylysine glycosides, 10% is free and unglycosylated, and the remainder is peptide-bound, which suggests that free hydroxylysine is largely metabolized and not excreted (76). Age influences these ratios. Free and peptide-bound hydroxylysine are most prominent in the urine of infants and children. Urinary hydroxylysine glycosides increase from 6 months of age (7.1 ± 1.3 μmol/day) to puberty when the highest values are observed (45 + 4.7 μmol/day) (76). Normal adults excrete more Glc.GHYL than GHYL (21.5 ± 1.4 *vs.* 13.7 ± 0.7 μmol/day). The Glc.GHyl/GHyl ratio is always higher in adults than in children, indicating a greater contribution of bone collagen in children due to their rapid growth and modeling (76). Recent studies have focused less on the glycoside ratio in the urine and more on the urinary level of GHYL, the more bone-specific glycoside, to identify individuals with increased rates of bone resorption (77, 78).

2. Methods. Analytical methods for GHYL are complex and time consuming. Early methods required clean-up steps, resolution using an amino acid analyzer, and detection of the hexoses by orcinol-sulfuric acid (71). In the latest method, urine is used directly. Hydroxylysine is dansylated and the fluorescent derivatives are resolved and detected by reversed-phase HPLC (79, 80). The necessary dansylated GHYL standard can be prepared from human urine (81). Commercial availability of such a standard may expand study and application of GHYL as a bone resorption marker.

3. Clinical correlates. Although results are promising, GHYL has not been widely studied or validated as a marker of bone resorption. Levels are elevated in children and Paget's disease patients (82). Differences in 24-h excretion between men and women were attributed to differences in body size and could be normalized to creatinine excretion or body surface area (76). In young girls midpubertal GHYL/creatinine levels were 4.02 ± 0.2 μmol/mol before menarche, approximately 5.3-fold higher than adult levels, and dropped to 3.1 ± 0.2 μmol/mol after menarche (83). Urinary GHYL/creatinine levels increase at menopause and correlate negatively with bone density in women (77, 78). In a study of 160 women, GHYL/creatinine levels also correlated with serum alkaline phosphatase, but not with urinary hydroxyproline/creatinine levels (77). Suppressed GHYL levels were noted in osteoporotic women in response to oral vitamin D, calcitonin, and rehabilitation exercise therapy (84).

In a comparison of urinary GHYL, urinary hydroxyproline, and serum alkaline phosphatase, GHYL was the only marker that could predict metastases in premenopausal women with breast cancer (85). The clinical performance of GHYL, hydroxyproline, and total pyridinolines as bone resorptive markers were recently compared (82, 86). All showed similar discriminating power in subjects with high resorption (Paget's disease and children). Only GHYL and the pyridinoline cross-links were able to discriminate postmenopausal osteoporotic women. While it is a promising marker of bone resorption, GHYL requires further validation against bone histomorphometry and calcium kinetics. The validity of using spot urines rather than 24-h urine collections also needs investigation.

C. Pyridinoline cross-links: total, telopeptide, and free forms

1. Biochemistry. In the last decade, collagen cross-links have evolved as the most promising markers of bone resorption. Pyridinoline (Pyr) and deoxypyridinoline (Dpy) residues originally received the most attention. Newly deposited collagen fibrils in the extracellular matrix are stabilized by cross-links formed by the action of lysyl oxidase on lysine and hydroxylysine residues in telopeptide domains of the collagen molecules. The resulting aldehydes condense with hydroxylysyl or lysyl residues on adjacent collagen molecules to form divalent cross-links, which can mature by further condensation with telopeptide aldehydes to the trivalent structures Dpy (also referred to as lysyl pyridinoline, LP) and Pyr (also refered to as hydroxylysyl pyridinoline, HP) as shown in Fig. 2.

Pyridinolines act as mature cross-links in types I, II, and III collagens of all major connective tissues other than skin (87, 88). This includes type I collagen of bone, dentin, ligaments, fascia, tendon, vascular walls, muscle, intestine, etc. In all tissues, Pyr predominates, with Dpy, the minor component. Dpy is found in highest concentration relative to Pyr in bone and dentin at a Pyr/Dpy ratio of 3.5:1 (22% Dpy) in human bone. The pyridinoline cross-links occur essentially at two intermolecular sites in the collagen fibril: two aminotelopeptides are linked to a helical site at or near residue 930 and two carboxytelopeptides to helical residue 87 (Fig. 2). The two helical sites are thus placed symmetrically at about 90 residues from the ends of the 1000-residue helical domain. Dpy is derived from two hydroxylysines and one lysine residue, while Pyr is derived from three hydroxylysine residues. The combined concentration of Dpy and Pyr in bone is only 0.3 mol/mol of collagen. Most nonmineralized connective tissues (other than skin) contain higher concentrations of the total cross-links (Table 2). When mature collagen is degraded, the peptide fragments will include those that contain pyridinoline cross-links. The products of collagen degradation containing the Dpy and Pyr cross-links in urine range in size from the free cross-linking amino acids to segments of the N-telopeptide and C-telopeptide sequences. These small peptides (<2 kDa) and free pyridinoline cross-linking structures are presumably readily cleared by the

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TABLE 2. Distribution of pyridinoline cross-links in human bone and joint tissues in moles/mole collagen ± SD[a]

	n	Pyr	Dpy	Pyr/Dyr Ratio
Bone, cortical	18	0.26 ± 0.09	0.07 ± 0.02	3.5:1
Bone, cancellous	18	0.18 ± 0.08	0.05 ± 0.02	3.5:1
Articular cartilage	18	1.48 ± 0.32	0.03	>40:1
Meniscus	24	1.40 ± 0.14	0.05	>20:1
Joint capsule	14	0.86 ± 0.17	0.07	>12:1
Annulus fibrosus	6	1.66 ± 0.14	0.03	>50:1
Nucleus pulposus	21	1.59 ± 0.44	0.03	>50:1

[a] Values are from Ref. 89 for bone and articular cartilage, and Eyre *et al.* (unpublished). The low content of pyidinolines in bone collagen reflects incomplete maturation of borohydride-reducible cross-links.

kidneys. To our knowledge, there is no current evidence that the pyridinolines themselves can be metabolically degraded.

Pyridinoline cross-links in urine are thought to originate primarily from bone resorption. This conclusion is supported by the observed similarity in ratio of Pyr/Dpy in adult human bone (22% Dpy) and urine (from 13–33% Dpy), compared with most nonbone connective tissues. In these tissues, Dpy is usually present at less than 10% of Pyr. The contribution to urinary Pyr and Dpy from soft tissue degradation has not been established. However, it should be noted that even though the Pyr/Dpy ratio is much higher in all other connective tissues, the actual concentration of Dpy in certain soft tissue collagens may be as high as it is in bone (88–91). When expressed as moles of Dpy/mol of collagen, vascular tissue and skeletal muscle have similar concentrations of Dpy as bone. In skeletal muscle, the ratio of Pyr/Dpy is only twice that in bone, and it is estimated that half the Dpy in urine could come from such nonbone sources (91). Although it is possible that soft tissues contribute to the normal excretion of Dpy and Pyr, bone represents the major reservoir of total collagen in the body and turns over faster than most major connective tissues. On the other hand, the contribution to urinary pyridinolines from nonbone sources in specific disease states, *e.g.* muscle atrophy, remains to be established.

Pyridinolines and cross-linked collagen peptides have several theoretical advantages over hydroxyproline as markers of bone resorption. They can only come from mature, extracellular collagen fibrils, not newly made molecules that are terminated at synthesis. Unlike hydroxyproline, the pyridinoline amino acids are fully excreted with no known pathway of metabolic degradation. Usually about two-thirds of the pyridinolines in urine are in the form of small peptides (<2 kDa). These peptides have sequences specific to the parent collagen molecules (*e.g.* collagen type I), providing a basis for added specificity as molecular markers of bone. Osteoclasts cultured on human bone particles generate cross-linked peptides but not free pyridinoline cross-links (92). Other tissues that contain type I collagen, such as skin, will also produce immunoreactive peptides *in vitro* (91, 93). It is not clear where in the body the peptides are broken down to yield the pool of free pyridinolines in urine (~30% of total) and whether bone collagen is the only source of the free pyridinolines. Similar to hydroxyproline, the pyridinolines are present in the diet, but unlike hydroxyproline, they appear not to be absorbed (93, 94). Therefore, no dietary restrictions are necessary, which is a major advantage.

2. *Methods.*

a. Total pyridinolines. The first and most applied method for measuring urinary Pyr and Dpy involves acid hydrolysis of the urine sample, solid-phase extraction, resolution by HPLC, and quantification of their natural fluorescence (87, 95, 96). Method variations include modifications of the chromatographic conditions and of the extraction method after acid hydrolysis (97–100). For total pyridinolines, the urine must first be hydrolyzed to the free amino acids. In most cases, equal volumes of urine and 12 M HCl are mixed and heated under standard protein hydrolysis conditions. This step is a potential source of destruction of pyridinolines and may contribute to the variability in results seen among laboratories (89). However, in a recent study, simply increasing the hydrolysis time (tested range 18–120 h) did not affect the recovery of total urinary Dpy and Pyr (94).

Total pyridinolines measured by the HPLC method are stable when urine is stored at −20 C in the dark for at least 18 months (94, 98). The cross-links are also stable to repeated (10 times) freezing and thawing cycles (101). However, other reports indicate a significant lability to both natural and artificial light, particularly UV wavelengths, of pyridinolines in urine samples exposed for even brief periods (102). Use of spot urine samples may be preferred over 24-h urine collections because of ease of collection, particularly in large survey studies. A strong correlation between pyridinoline cross-links in spot urines and 24-h collections was found in most (94, 103, 104), but not all, studies (105).

A basic problem in comparing results of the HPLC method among laboratories is the lack of a common reference standard. A variety of external standards of undefined purity have been used, including Pyr and Dpy isolated from bovine (98), ovine (95, 103), canine (101, 106), and human bone (99, 100, 107, 108) or urine from patients with Paget's disease of bone (94). The HPLC method needs to be standardized among laboratories before fully meaningful comparisons of results can be made. Assay precision has been improved using internal standards such as acetyl Pyr, a semisynthetic derivative (109, 110), or other fluorescent products prepared from elastin (94). Inter- and intraassay CVs for the HPLC technique differ greatly among reporting laboratories but are usually both less than 15%. Use of an internal standard lowered interassay CVs from 15.1% to 5.3% for Pyr and from 20.8% to 4.6% for Dpy (109).

b. Free pyridinolines. Clinical application of pyridinoline assays has been limited by the inconvenience of the HPLC method. Although an early enzyme linked immunosorbent assay (ELISA) was developed for Pyr in urine, it required acid hydrolysis before assay (111). Recently a polyclonal antibody-based ELISA that recognizes the free Pyr and Dpy in urine was reported, which correlated well with total Pyr measured by HPLC. This antibody does not distinguish between Pyr and Dpy or recognize peptide-bound Pyr to a significant degree. Studies have reported intra- and interassay variations for free Pyr below 10% and 15%, respectively, and a sensitivity of 25 nM (103, 112–114).

Because of the abundance of Pyr in tissues other than bone, a selective immunoassay for Dpy should be more specific as a bone-resorption marker. An antibody- based ELISA and an RIA (115–117) for Dpy have been developed. For the ELISA,

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Robins *et al.* (116) report less than 1% cross-reactivity with free Pyr and no interaction with peptide forms of the cross-link. The intra- and interassay variations were less than 10% and 15%, respectively, and the assay sensitivity is 2 nM Dpy.

The availability of these assays in a commercial form (Pyrilinks, Collagen Crosslinks Immunoassay and Pyrilinks-D, Deoxypyridinoline Immunoassay, Metra Biosystems, Palo Alto, CA) has advanced the clinical research application of these urinary resorptive markers.

c. Cross-linked telopeptides. Because the pyridinolines are known to be excreted predominantly in peptide-bound form, several laboratories have developed assays for pyridinoline-containing peptide fragments. One monoclonal antibody based-ELISA was developed that recognizes a discrete pool of cross-linked N-telopeptides of collagen type I isolated from urine (118). This region was chosen because the Pyr/Dpy ratio in this peptide pool indicated an origin in bone. (Two-thirds of Dpy in bone collagen is at the N-telopeptide and only one-third at the C-telopeptide site.) The monoclonal antibody recognizes a peptide conformation in the α2(I)N-telopeptide. This implies further bone specificity since the pyridinoline cross-link in bone primarily involves the α2(I) chain whereas in other tissues the α1(I) chain predominates. The antibody does not recognize the uncross-linked linear telopeptide precursor, free pyridinolines, or the pyridinoline residue itself (118). The analyte, NTx, contains the cross-linked α2(I)N-telopeptide sequence, *QYDGKGVG*, which is a product of osteoclastic proteolysis and in which K is embodied in a trivalent cross-linkage (91). Collagen must be broken down to small cross-linked peptides that contain this exact sequence before the antibody can bind to the NTx antigen. The antibody also recognizes such peptides in culture medium conditioned by osteoclasts that are resorbing human bone particles *in vitro* (91, 92). This suggests that the NTx peptide is a direct product of osteoclastic proteolysis, does not require further metabolism in the liver or kidney for generation, and is rapidly cleared by the kidney.

The NTx-ELISA requires no hydrolysis or pretreatment of the urine and is available in a commercial, microtiter-plate format (Osteomark, Ostex International Inc., Seattle, WA). The assay measures the NTx peptide analyte in spot urines, calibrated in molar equivalents of bone type I collagen. Results are expressed normalized to creatinine. The calibration curve is based on standards prepared from bacterial collagenase-digested human bone collagen. Studies using this commercially available NTx-ELISA report inter- and intraassay CVs of less than 10% and a sensitivity of 20 nM.

Another ELISA for measuring type I collagen telopeptides in urine has recently been introduced in commercial form (CrossLaps, Osteometer, Rodrove, Denmark) and evaluated in healthy pre- and postmenopausal women and several disease states (119, 120). This assay is based on a polyclonal antiserum raised against a synthetic eight-amino acid peptide (*EKAHDGGR*) having an amino acid sequence that matches a segment of the C-telopeptide of the collagen α1(I) chain and is referred to as CTx. The assay developers chose this particular sequence of amino acids as the antigen because it contains the lysine of the C-telopeptide domain that participates in intermolecular cross-linking and because they anticipated that it would be protected from degradation when embodied in pyridinoline-containing structures excreted into urine (120). This C-telopeptide structure is common to all tissues in which type I collagen is cross-linked by pyridinoline. Such domains, when part of compact, cross-linked peptides, were proposed to resist degradation in the kidney (118). Users of the commercial assay, CrossLaps, report intra- and interassay CVs of less than 10% and 13%, respectively, with sensitivity of 0.5 μg/ml (119).

An immunoassay that is applied to serum rather than urine has also been developed for the C-telopeptide cross-linking domain of human type I collagen (ICTP) (121). Results from the clinical application of this assay are disappointing and suggest that it lacks needed specificity as a marker of bone resorption. The assay uses a polyclonal antiserum raised against a purified cross-linked fragment prepared by digesting human bone collagen with bacterial collagenase and trypsin. The RIA for use in serum is available from Orion Diagnostica (Espoo, Finland and Incstar, Stillwater, MN). Studies using this commercial immunoassay report nonspecific binding of approximately 10%, intra- and interassay CVs of 5–8% and 6–9%, respectively (122, 123), and a detection limit of 0.34 μg/liter (121). A listing of all the commercially available assays of bone resorption are given in Table 3.

3. *Clinical correlates.* It is difficult to compare results from the clinical application of these various methods because different analytes and different units are used and they may each reflect the consequences of distinct biological processes. To avoid confusion, therefore, the clinical results will be discussed separately for each of the above analytical approaches.

a. HPLC assay. Urinary excretion of total pyridinolines (free plus peptide-bound Pyr and Dpy) measured by HPLC varies with age, *i.e.* higher levels in children than adults. Mean values (±SD) for children normalized to creatinine were 372 ± 74 and 106 ± 21 nmol/mmol for Pyr and Dpy, respectively, and 27.2 ± 19 and 8.8 ± 0.8 nmol/mmol, respectively, for adults 21–70 yr of age (98). From group data, the Pyr:Dpy ratio in urine does not appear to vary with age or gender in adults, although significant variations among individuals are evident. This was also noted when individual samples of human bone were analyzed for these cross-links (88). After skeletal growth ceases, excretion is relatively constant, but in women increases again after menopause (105, 106, 124, 125) and is reported to gradually increase further until the ninth decade (126). Estrogen replacement restores urinary levels of Pyr and Dpy to premenopausal values (105).

Studies using the HPLC method report individual day to day variations in urinary excretion of 16–26% (94, 104). A circadian rhythm in urinary excretion of both Pyr and Dpy normalized to creatinine was reported, with peak excretion in the early morning (0300–0800 h) and a nadir in the afternoon to late evening (1400–2300 h) (127, 128). Nocturnal increases in urinary Dpy of 10% for men (104) and 48% for postmenopausal women (129) have also been reported. This latter study found that postmenopausal women with osteoporosis excreted high levels of Dpy during both day and night and with no pronounced nocturnal increases (129). With spot urines or limited collections, therefore, the time of

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TABLE 3. Commercially available assays for resorptive markers of bone turnover

Assay name	Manufacturer	Analyte	Assay method	Sample requirements[a]	Sensitivity
Hypronosticon	Organon Teknika, Inc., Scarborough, Ontario, Canada; Boxtel, Holland	Free and peptide-bound hydroxyproline	Colorimetric	0.5 ml urine (24 h urine collection); dietary restrictions, collagen free	
Pyrilinks	Metra Biosystems, Inc., Mountain View, CA	Free Pyr and free Dpy	EIA	100 μl urine (first morning void, 24 h or second morning void); no diet restrictions	7.5 nM
Pyrilinks D	Metra Biosystems, Inc., Mountain View, CA	Free Dpy	EIA	100 μl urine (first morning void, 24 h or second morning void); no diet restrictions	1.1 nM
Total Dpy	Nichols Institute Diagnostics, San Juan Capistrano, CA	Total Dpy	RIA	0.5 ml urine (store in amber container)	0.06 nmol/liter
Free Dpy	Nichols Institute Diagnostics, San Juan Capistrano, CA	Free Dpy	RIA	0.5 ml urine or 0.1 ml serum or EDTA plasma	0.06 nmol/liter
Osteomark	Ostex International, Inc., Seattle, WA	Cross-linked N-telopeptide of type I collagen	ELISA	25 μl urine (second morning void or 24 h urine collection)	20 nM BCE/liter[b]
ICTP	Incstar Corporation Stillwater, MN; and Orion Diagnostica, Espoo, Finland	Cross-linked C-telopeptide of type I collagen	RIA	100 μl serum	0.5 μg/liter
Crosslap	Osteometer Rødovre, Denmark; and Diagnostic System Laboratories, Webster, TX	Cross-linked C-telopeptide of type I collagen	ELISA	25 μl urine (fasting second morning void)	0.2 mg/liter

[a] Sample requirements are per tube or well; assays usually require samples to be run in duplicate or triplicate.
[b] Bone collagen equivalents.

sampling requires careful attention to minimize the potential confounding effects of circadian rhythms. The contribution of creatinine rhythmicity to the observed circadian variations in urinary markers may also be a dominant factor as a recent study indicates (130).

Pyr and Dpy levels in urine correlate with histological measures of bone turnover from bone biopsies (131) and with radioisotopically determined bone resorption (132). Increases were seen in diseases known to accelerate bone turnover, including primary hyperparathyroidism (103), Paget's disease (99), rheumatoid arthritis (133), osteoporosis (57, 131, 134, 135), and hyperthyroidism (136, 137). The ability of urinary Pyr and Dpy to monitor antiresorptive therapies has also been explored. Bisphosphonate therapy for increased bone resorption in metastatic bone disease suppressed urinary Pyr and Dpy markedly (107, 108). Compared with total serum alkaline phosphatase and urinary calcium, urinary Pyr was best able to discriminate between cancer patients with and without bone metastases (138). Urinary Dpy was suppressed in subjects who received salmon calcitonin intraperitoneally (139) but not in those who received it by suppository (140). Total urinary Dpy measured in postmenopausal osteoporotic women by HPLC was suppressed by bisphosphonate treatment to the premenopausal range, and the percent change from baseline correlated with the increase in spinal bone mineral density for the group on therapy (141).

b. Immunoassays for free Pyr and free Dpy. The free Pyr ELISA has shown increases in normal infants that correlated with growth velocity (142). Urinary excretion of free Pyr measured by ELISA increased with adult age in both men and women but was higher in women (112, 113). Women showed higher mean values after menopause compared with a group of younger premenopausal women (43 *vs.* 59 nmol/mmol creatinine) (113). Correlation coefficients of ELISA results for free Pyr with total Pyr by HPLC vary significantly from high ($r = 0.82$ to 0.97) to moderate ($r = 0.67$ to 0.79) depending on the subject group. When correlations include very high turnover states (Paget's disease, hyperparathyroidism), the correlation is higher than with a more homogeneous group of subjects with relatively low turnover (112–114). It was also reported to be a useful monitor of bone resorption in assessing the activity of Paget's disease (143).

Recent clinical studies in which free Pyr was compared with other markers raise questions on the specificity of this analyte to bone and the resorption process. In a study by Garnero *et al.* (141), a comparison of premenopausal women (mean age 40 yr, n = 46) and postmenopausal women of determined low bone mass (mean age 63 yr, n = 85) found that both total Pyr and total Dpy by HPLC and free Pyr by ELISA were elevated in the postmenopausal group. In assessing the response to bisphosphonate therapy in the same study, however, free Pyr showed no significant suppression despite highly significant decreases of 30% in total Pyr and 50% in total Dpy by HPLC. In another short-term study of bisphosphonate suppression of bone resorption in young adult males, free Pyr also proved to be relatively unresponsive compared with other markers (144). Results of the same study, using thyroid hormone (T_3) to stimulate metabolism, implied that other tissues or metabolic influences might be contributing to urinary free Pyr. While free Pyr appears to be insensitive to antiresorptive agents such as bisphosphonates in osteoporosis, in Paget's patients treated with bisphosphonate, free Pyr was suppressed but less markedly than total pyridinolines or the cross-linked telopeptides (145). Free Pyr was sensitive to changes in estrogen status in postmenopausal women (119, 141, 146).

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The relative insensitivity of free Pyr could be explained if nonosteoclastic pathways are responsible for generating the free amino acid from peptide products of collagen degradation in the body. A greater contribution from nonosseous tissues, which also turn over faster in such disease states as hyperthyroidism and malignancy, has also been proposed (91, 147). Despite these limitations, the convenience and ease of the free Pyr ELISA assay may make it useful for certain clinical applications, *e.g.* in monitoring disease activity in Paget's disease or as a convenient marker for growth in normal infants and children. It merits further study and cautious consideration, however, when applied to osteoporosis studies.

There is only one report of a serum assay for free Pyr. With this assay, significantly higher serum levels were reported in dialysis patients with high turnover bone disease, which correlated with both dynamic and static parameters of bone histomorphometry (148).

Only a few studies on the more recent ELISA for free Dpy in urine have been published. A 5-fold higher excretion rate in children than adults and higher levels in primary hyperparathyroidism, hyperthyroidism, and Paget's disease compared with premenopausal women are reported (116, 119). As with free Pyr, free Dpy levels failed to respond to short-term bisphosphonate therapy but were lowered by long-term estrogen treatment (119). Full clinical evaluation of this assay awaits the publication of results from several ongoing human studies.

c. Immunoassays for cross-linked N- and C-telopeptides of collagen type I in urine. These analytes are referred to as NTx and CTx. A growing number of basic and clinical studies have reported NTx results (118, 128, 130, 141, 144–146, 149–154) and CTx results (120, 147, 155) in various clinical conditions. Two studies have compared the results of both these telopeptide assays and other bone markers (119, 145).

In summary, the cross-linked telopeptides in urine show a greater increase at menopause than pyridinolines (total or free) or other resorption markers and a greater degree of suppression when used to monitor the effects of the antiresorptive agents, bisphosphonates and estrogen (119). The simplest molecular explanation is that the cross-linked telopeptides in urine are more specific products of bone, in particular the proteolytic process of bone resorption brought about by osteoclasts (91), than are either total or free pyridinolines or hydroxyproline. Alternatively, it has been argued that bisphosphonates, but not estrogen, may alter the degradation pattern of bone collagen by osteoclasts so that the ratio of free pyridinolines to peptide pyridinolines is altered (119). Effects of bisphosphonates on kidney clearance have also been suggested (145). The full explanation may include elements of all these concepts, which are not mutually exclusive, but in time, no doubt, new data will resolve the issue.

Cross-linked N-telopeptide (NTx) levels in urine are highest in infancy and childhood (118), show a peak at the primary growth spurt in boys and girls (130), and fall to a plateau in adulthood with a rise after menopause in women (141, 156). Early postmenopausal women (6 months to 3 yr post menopause) show a mean NTx/creatinine ratio 2- to 3-fold that of age-matched or younger premenopausal women (141). In bisphosphonate-treated patients, mean levels were suppressed up to 70% with a dose dependence (141, 150). Young adult males showed an even higher percent suppression of NTx from baseline when given short-term pamidronate intravenously (144). The suppression of bone resorption in Paget's disease patients treated with bisphosphonates has also been monitored effectively (118, 153). Other pathological conditions in which urinary NTx is elevated include acromegaly (149), hyperthyroidism (119), and metastatic malignancies (157).

Fewer independent studies have been reported on CTx (CrossLaps assay from Osteometer), but, similar to NTx, large postmenopausal increases and large decreases are observed after bisphosphonate and estrogen therapy (119, 120, 145). Compared to controls, CTx appeared to differ from NTx when both analytes were measured on the same samples. Relative to NTx, CTx was less elevated in Paget's disease patients and more elevated in hyperthyroid patients, suggesting greater bone specificity of NTx (119).

d. Immunoassay for C-terminal telopeptides of type I collagen in serum. Serum levels of ICTP measured by immunoassay correlated well with histomorphometric indices of bone resorption and formation from iliac crest biopsies from patients with disorders of high and low bone turnover (123). The normal range of serum ICTP is reported to be 1.5 to 4.2 μg/liter, and disorders such as osteolytic metastases can show 20-fold increases. Serum ICTP shows a circadian rhythm, with 20% higher levels at night (0200–0550 h) than in the afternoon (123, 158), similar to the rhythms of osteocalcin, pyridinoline cross-links, NTx peptides, and hydroxyproline, which all exhibit nocturnal increases.

Serum ICTP levels increased about 20% after menopause but decreased only a modest 10% after 1 yr of hormone replacement therapy (159). Consistent with this, ICTP levels correlated with an index of calcium kinetics (retention of 99 m-technetium diphosphate), but not with histomorphometric measures of turnover in bone biopsies from postmenopausal women with mild osteoporosis (160).

This marker was also elevated in primary hyperparathyroidism and after PTH infusion (161). However, in monitoring bisphosphonate treatment in late postmenopausal osteoporosis, ICTP did not change after treatment. Neither baseline serum levels nor changes in ICTP correlated with bone mineral density changes (141). These findings support a conclusion that while serum ICTP measurement may detect changes in collagen metabolism due to disease or menopause, it is not a sensitive or specific marker of bone resorption that responds to hormone replacement therapy or can monitor the effects of other antiresorptive agents.

D. Tartrate-resistant acid phosphatase (TRAP)

During active bone resorption, osteoclasts secrete acid and enzymes into the space between the ruffled border of the osteoclast and the surface of the bone. The enzyme, TRAP, has been identified in both the ruffled border of the osteoclast membrane and the secretions in the resorptive space (162). Measuring TRAP activity in serum should, therefore, serve as an accurate index of osteoclast activity and bone resorption (163–167). While TRAP is promising, far less attention

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has been paid to TRAP because of the difficulty in measuring the isoenzyme unique to osteoclast activity.

1. *Biochemistry.* The group of acid phosphatases to which TRAP belongs is widely distributed with the greatest concentrations in prostate, liver, kidney, erythrocytes, platelets, and osteoclasts (163). These enzymes are nonspecific hydrolases that hydrolyze phosphomonoesters at low pH (166). Elevated serum acid phosphatase activity is clinically significant in the diagnosis of prostatic disease, since acid phosphatase activity is 1000-fold greater in the prostate than in other tissues. Serum acid phosphatase activity from the prostate is inhibited by tartrate. Early methods, therefore, used tartrate inhibition as a basis for more specific assays of the prostatic enzyme and diagnosis of cancer and other disorders of the prostate.

Six isoenzymes (types 0–5) of acid phosphatase have been identified by electrophoresis of human tissues (167). The isoenzyme expressed by osteoclasts is identical to the serum type 5b isoenzyme (168), but other tissues also express this component (169). All the band 5 acid phosphatases are resistant to tartrate inhibition (169–172), but other classes of acid phosphatases also have this property, *e.g.* erythrocyte. Serum band 5 TRAP activity is normally expressed by osteoclasts, alveolar and monocyte-derived macrophages, and the placenta (173). The spleen also shows activity in Gaucher's disease and in hairy cell leukemia (169).

Unlike bone alkaline phosphatase, little is known about tissue-related posttranslational modifications of TRAP. In general, TRAPs are cationic glycoproteins of 30–40 kDa that contain two atoms of iron and are thought to be the product of a single gene (165–168). Recently Allen *et al.* (166) reported the purification and characterization of TRAP from human bone and produced immunopurified monospecific rabbit antibody to human bone TRAP. These advances show promise for the development of a bone-specific immunoassay for TRAP activity in human serum.

2. *Methods.* Difficulty in distinguishing the osteoclastic enzyme in serum from other tissue acid phosphatases presents the greatest technical barrier. TRAP activity in serum has been measured kinetically (168–172) and, more recently, by immunoassay (173–178), but these methods vary in specificity for the osteoclast-derived isoenzyme and need to be validated. Kinetic assays are available commercially [Bio-Merieux, Charbonniè-les-Bains, France (83) and Sigma, St. Louis, MO (135)]; however, these assays measure TRAP activity and are not specific for the bone isoenzyme. In order to make meaningful comparisons between methodologies and/or laboratories in the use of TRAP to monitor metabolic bone disease, more studies are needed.

The most widely used kinetic assay (168) spectrophotometrically monitors the hydrolysis of *p*-nitrophenyl phosphate in the presence of sodium tartrate. In this assay the interference by serum factors that act as noncompetitive inhibitors of TRAP is avoided by diluting the serum and increasing the substrate concentration. Interference by erythrocytic TRAP activity released by hemolysis is reduced by incubating the serum at 37 C for 1 h before assay. The procedure of Lam *et al.* (174) immunoprecipitates the band 5 acid phosphatases and then measures their enzyme activity kinetically. Kinetic assays are least specific for the osteoclast enzyme and most susceptible to changes in serum stability. The enzymatic activity of TRAP requires the presence of iron, which can be supplied by transferrin. TRAP activity is also sensitive to oxidizing and reducing agents. Lower serum levels of TRAP were observed when α-naphthyl-phosphate was used as a substrate (172).

Immunoassays for TRAP measure the protein concentration rather than its enzyme activity and resistance to tartrate. However, most applied immunoassays (173, 175) were not developed using osteoclast TRAP. The earliest assays used antisera raised against uteroferrin, which resembles osteoclastic TRAP (169, 176). They showed marked improvement over the kinetic assays and less interference from the non-band 5 class of acid phosphatases. More recently, Kraenzlin *et al.* (175) developed an ELISA with antibodies raised to TRAP isolated from spleen cells of patients with hairy cell leukemia, whereas Cheung *et al.* (173) used TRAP from human cord plasma. Both ELISAs showed promise as assays for bone-specific acid phosphatase as they cross-reacted with extract of bone, but not with extracts of normal spleen, erythrocytes, platelets, osteoblasts, or prostrate. Serum levels were also appropriately high in children, postmenopausal women, and in conditions of increased bone turnover (169, 173). However, the enzymes from cord blood and hairy cells of the spleen need characterizing in comparison with osteoclastic TRAP. A purified source of osteoclast-derived TRAP is needed as a reference standard to calibrate these immunoassays and compare their results.

3. *Clinical correlates.* Several studies show the potential of measuring TRAP in serum to assess bone resorption activity. Using kinetic methods (168) TRAP can distinguish normal subjects from patients with skeletal diseases including Paget's, primary hyperparathyroidism, metastatic cancer, hypoparathyroidism, advanced renal failure, involutional osteoporosis, and osteomalacia (168, 179, 180). TRAP activity was inversely correlated with bone mineral density of the radius and lumbar spine (180), metacarpal cortical thickness, and bone mass of the lumbar spine (64) in postmenopausal women. TRAP and bone mineral content were inversely related in postmenopausal women with osteoporosis (181). TRAP activity is also elevated in growing children (182). TRAP activity paralleled changes in three other bone markers in urine during and after 7 days of immobilization (66, 183).

Neither kinetic assays nor immunoassays have been fully validated against traditional indices of bone resorption such as histomorphometry or radio-calcium kinetics. The immunoassay against human bone TRAP (166) did correlate with histological parameters of osteoclast activity in bone biopsies from renal dialysis patients (177, 178). The newer immunoassays distinguished between normal individuals and those with high bone turnover conditions, including Paget's disease and hyperparathyroidism, hyperthyroidism, and renal failure (173, 175, 184, 185). These ELISAs showed serum TRAP differences between children and adults, and between postmenopausal and perimenopausal women. TRAP activ-

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ity decreased in menopausal women receiving estrogen replacement therapy (173). Immunoassays of TRAP protein rather than activity also need to be validated using histomorphometry and calcium kinetics in larger study groups, and their ability to monitor antiresorptive agents needs to be established.

III. Biochemical Markers of Bone Formation

A. Serum alkaline phosphatase (ALP)

Serum ALP is one of the most frequently performed assays in clinical medicine. Its elevation in various skeletal disorders has been recognized for 60 yr. Changes in total ALP activity are useful as a therapeutic marker in Paget's disease, rickets and osteomalacia, renal osteodystrophy, and for monitoring the healing of new fractures but are less useful in osteoporosis and other disorders of bone. Most requests for fractionation of ALP result from the need to distinguish between liver and bone as alternative or coexisting sources when there is an unexplained elevated total ALP. On the other hand, an ALP within the normal range may obscure an abnormal isoenzyme pattern. Clearly the usefulness of ALP as a marker of bone activity depends on the ability to quantitatively distinguish the activity of the bone isoform from that of other tissues. Several recent reviews detail the biochemistry and clinical relevance of ALP from all sources (186–191).

1. Biochemistry. Total ALP in serum includes several isoforms. Elevated values result from increased activity of intestinal, spleen, kidney, placental, liver, bone, or expression by tumors. Germ cell ALP has low activity in its tissues of origin (testis, thymus, and lung) but increases with development of germ cell tumors (192). The ALP molecule from these different tissues varies in size, charge, and kinetic and physical characteristics but displays the same broad catalytic properties, *i.e.* the liberation of phosphate. Four gene loci code for ALP: the tissue-nonspecific (*tns*) gene, which is expressed in numerous tissues (including bone, liver, kidney, and early placenta), is located on the short arm of chromosome 1 (193); the other three tissue-specific genes encode the ALP of the intestine, mature placenta, and germ cell enzymes. They are closely linked and located on the long arm of chromosome 2 (194). The close proximity of these alleles suggests a recent gene duplication and divergence from a common ancestral gene (188).

The *tns* gene has longer introns and is 5 times longer than the other ALP genes. The proteins are similar in length (507–513 amino acids) and contain a 17- to 21-amino acid signal peptide. The sequence homology among the three tissue-specific gene products (intestine, mature placenta, and germ cell) ranges from 86–98%, whereas the homology between the tissue nonspecific ALP and the others is approximately 50% (188, 195). The catalytic sites are considerably conserved. Two zinc and one magnesium ion are bound in close proximity to each other in the active site region in all the isoforms (186). Tissue nonspecific ALPs are the products of a single structural gene. However, tissue-specific differences are found in their electrophoretic mobility, stability to heat, and sensitivity to a variety of chemical inhibitors. These differences are due to variations in their carbohydrate side chains and degree of sialation.

ALP is a member of a large group of proteins that are anchored to glycosyl-phosphatidylinositol moieties on the extracellular surface of cell membranes (196). As such, it is an "ecto-enzyme," expressed and functioning on the outside of the cell. The enzyme is a tetramer when membrane-bound but circulates as a dimer (197). Phospholipase C or D (which is abundant in plasma) potentially converts the membrane-bound form to a soluble form (198, 199)(Fig. 4). Other high molecular weight fractions comprised of liver ALP attached to membrane fragments (fast-liver ALP) also circulate. These are elevated in patients with hepatobiliary disease, being produced by the detergent action of bile acids (190, 191). Increases in serum ALP activity reflect increased translation of mRNA, but it is not known whether changes in phospholipase activity or the physical state of the phospholipid bilayer influence levels of ALP activity in particular disease states (199, 200).

ALP is found in most species from bacteria to man, but its physiological role is unknown. It most tissues, ALP is expressed in low levels, and it has been suggested that it serves a "house-keeping" function in these tissues (195). However, the protein is overexpressed in bone where it is thought to have a specific function involved with mineralization (201). Cells that express the *tns* gene at both low and high levels have similar transcription rates, initiate mRNAs from the same promoter, are equivalently spliced and transported from the nucleus, and have equal cytoplasmic stability. It appears that the expression of ALP is controlled at a very early posttranscription step by sequences within the gene's introns, possibly by destabilization of the nuclear RNA (202).

An essential role for the ALP enzyme in bone mineralization is evidenced by the disease hypophosphatasia, a rare inherited autosomal recessive disorder of osteogenesis characterized by defective mineralization of bones and teeth. The biochemical hallmark of the disease is deficient *tns*-ALP activity. No metabolic consequences in liver or kidney have been identified, and activities of the tissue-specific isoenzymes are normal. Clinical severity is variable, ranging from intrauterine death to fractures occurring in adulthood (201). In five cases of severe hypophosphatasia, mutations were identified as single point missense mutations in the conserved regions of the peptide (203, 204).

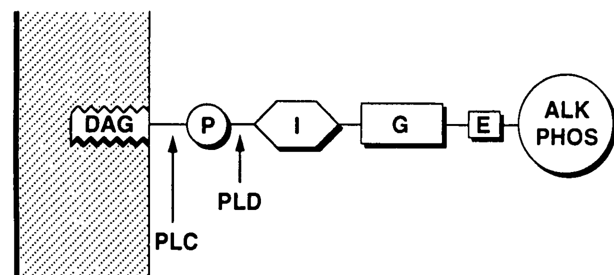

FIG. 4. Schematic representation of the anchoring of ALP in the plasma membrane. ALP is anchored to the cell membrane lipid bilayers [1, 2,diacyl-glycerol (DAG)] by a phosphatidylinositol-glycan (GPI) moiety through an ethanolamine that is amide linked to the carboxy terminus of the enzyme. The action of phospholipase C or D (which is abundant in plasma) potentially converts the membrane-bound form to a soluble form.

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Several possible roles for ALP in bone formation have been proposed. It may increase local concentrations of inorganic phosphate, destroy local inhibitors of mineral crystal growth, transport phosphate, or act as a calcium-binding protein or Ca^{2+}-ATPase. In any of these cases, the high lateral mobility of the enzyme attached to its glycosyl-phosphatidylinositol anchor may be important to its function (196). *In vitro*, actively proliferating osteoblasts produce an extracellular matrix of fibronectin and type I collagen but only low levels of ALP. At confluence, ALP activity increases and mineralization soon follows (205). Phospholipase C inhibits mineralization of these cultures (206, 207). In human osteoblast-like cells ALP activity is proportional to inorganic phosphate concentration, and the release of the enzyme from its phospholipid anchor is inversely proportional to calcium concentrations (208, 209). Together, these data suggest that the enzyme may participate in the initiation of mineralization; however, no definitive function has been established.

2. *Methodology.* The tissue nonspecific enzymes have identical protein primary structures but differ in composition of their carbohydrate side chains. Since the two most common sources of elevated ALP levels are liver and bone, a number of techniques have been developed that rely on these differences to distinguish between bone and liver isoforms. These include heat denaturation, chemical inhibition of selective activity, gel electrophoresis, precipitation by wheat germ lectin, and immunoassays.

The heat denaturation method is based on the gradation in heat stability at 56 C of the ALP enzymes found in serum, which ranges from placental (completely heat stable) to liver ALP, which has intermediate stability (half life = 7.6 ± 1.5 min), and bone ALP, which is very labile (half life = 1.9 ± 0.4 min). In this method, serum is heated to 56 C for 10 min and immediately cooled, and activity is measured in heated and unheated serum. When activity in heated serum is 20% or less of that in unheated serum, ALP is attributed to the bone enzyme, while heat-stable activity of 25–55% is attributed to the liver enzyme. This method demands precise temperature control throughout the assay. The inclusion of internal standards of skeletal and liver ALP activity allows for calibration of rate of inactivation (210), but precise quantification is difficult when high concentrations of both liver and bone enzymes are present or when ALP originates from placenta or intestine.

Another common method for distinguishing among ALP isoenzymes is by PAGE. Liver ALP carries the highest net negative charge, followed by the placental, bone, and intestinal forms. Liver and bone ALP can be separated sufficiently to allow visual assessment of their relative proportions, but these methods are tedious and there is often overlap between the two, making precise quantification difficult (189). Several methods have used selective inhibition to improve the separation (211, 212).

Wheat germ lectin binds to *N*-acetylglucosamine and sialic acid residues and provides a method by which to separate liver and bone ALP. Based on their differing glycosylation patterns, wheat germ lectin selectively binds the bone form (213, 214). Proper standards and lectin concentrations are necessary for accuracy. The lectin must precipitate more than 95% of authentic bone ALP and less than 5% of the liver isoform (214–216). Several studies have used serum samples as standards, which contain primarily one form of the enzyme. Serum from patients with liver disease can be used as liver standards, but serum from Paget's patients, which has been used as a bone standard, is not always suitable because of variable liver contribution. In studies in which serum from Paget's patients was used to determine the quantity of lectin needed for maximum precipitation of the bone isoform, skeletal and hepatic ALP were poorly resolved, and heat inactivation performed better than wheat germ lectin precipitation (217). Cord blood is a better bone standard because it contains only the bone isoform, the liver enzyme being absent from cord blood. When cord blood was used as an assay calibrator, values obtained by lectin precipitation and heat inactivation agreed (218). Controlling lot-to-lot variation in the lectin, reconstituting the lectin immediately before use, and treating the samples with detergent (which converts any potential biliary ALP to liver ALP, see below) has also improved accuracy and reproducibility (216, 219). A commercial kit using precalibrated lectin is available (Iso-ALP, Boehringer-Mannheim, Mannheim, Germany). Several investigators have also included wheat germ lectin in electrophoresis equilibration buffers to improve resolution (220, 221).

Several recent attempts to produce tissue-specific monoclonal antibodies have resulted in antibodies with preferential, but not exclusive, recognition of the liver form (222, 223). Antibodies with a 2- to 5-fold preference for liver ALP over the bone isoform have been used in indirect methods to estimate bone ALP, but the results have been disappointing. Two commercial kits that utilize monoclonal antibodies with preference for the bone isoform are currently available (224–225). Alkphase-B (METRA Biosystems, Mountain View, CA) uses a single monoclonal antibody made to purified ALP from a human osteosarcoma cell line. The antibody, bound to a microtiter plate, captures ALP from the sample, and activity of the bound enzyme is measured directly. Cross-reactivity to the liver form is about 10%, and there is a high correlation (r = 0.99) between this kit and values obtained with wheat germ lectin precipitation. In patients with Paget's disease and osteomalacia, mean bone ALP values were 8 and 2 times the upper limit of normal, respectively. In patients with osteoporosis and primary hyperparathyroidism, however, 23/32 and 17/20 patients, respectively, fell within the normal range (225).

A two-site immunoradiometricassay (IRMA) (Tandem-R-Ostase, Hybritech, San Diego, CA) relies on the use of two monoclonal antibodies, both of which react preferentially with the bone isoform. Cross-reactivity with the liver ALP is 16% (226). Good correlation between this immunoassay and agarose gel electrophoresis have been demonstrated ($r >$ 0.9)(226–228). Variable correlations are found between this kit and wheat germ lectin precipitation. In one study wheat germ lectin was calibrated with cord blood, and a good correlation was found (r = 0.87)(229), while a second study used the commercial wheat germ lectin kit and a poorer correlation resulted (r = 0.67)(230). We have found a good correlation (r = 0.83, n = 120) between the Tandem-R-Ostase kit and wheat germ lectin methods when our lectin was

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standardized against cord blood. (C. Gundberg, unpublished observations). In patients with significant elevations in total ALP (> 2-fold the upper limit of normal) cross-reactivity between bone and liver may lead to falsely elevated increases in skeletal ALP. In one study, 14 of 15 samples with electrophoretic patterns indicating hepatic disease and two of three with intestinal patterns gave elevated Ostase results (227). It remains to be determined, with the various antibody-based commercial kits, whether protein concentration or enzymatic activity provides the more accurate assessment of bone status. A listing of all the commercial kits available for bone formation markers is given in Table 4.

There are important caveats to be considered when using any of these methods for ALP determination. 1) ALP of intestinal origin is elevated after a fatty meal in normal people of blood types B or O (~80% of the population)(231). For methods that do not quantitatively separate the intestinal from the bone and liver isoforms (heat inactivation and chemical inhibition), accuracy is improved with fasting blood samples. 2) Preparation of ALP from tissue sources often yields membrane-bound enzyme (232), and there may be glycosylation heterogeneity within tissues (233). Therefore, ALPs prepared from organ sources for use as standards or controls may be different from their corresponding forms in serum. Furthermore, altered glycosylation forms are present in disease states and particularly in malignancy, and the tumor-producing forms can coprecipitate with the bone form with wheat germ lectin (190). Extracts of human bone from neonates and patients with Paget's disease or osteosarcoma have demonstrated a range of glycosylation patterns (209, 232, 234). Finally, intrahepatic and bone ALP bind similarly to wheat germ lectin (235), but membrane-localized glycosyltransferases modify the hepatic ALP before it reaches the circulation. In children less than 6 months old, the glycosyltransferases may be immature, and variable amounts of this "pseudo-bone" form reach the circulation (220). This may also occur in hepatitis (214). For all of these reasons, when high bone ALP values are found with any method, a second test (*e.g.* 5'-nucleotidase) should be employed to rule out liver involvement. To confirm bone involvement, additional determinations of bone formation should be employed (*e.g.* osteocalcin or PICP).

3. Clinical correlates. The major factors that modify ALP activity are age, sex, and hormonal status (puberty or menopause). From birth to 6 weeks, both bone and intestinal ALP increase. No liver ALP is observed until 6 months of age (220). In children a wide range of ALP activity exists and correlates with height and weight, and until puberty the bone isoenzyme represents 77–87% of the total (232). Activity increases in children around the age of puberty, the maximum being earlier in girls than in boys, and corresponds temporally with growth spurts in both sexes (220). A gradual increase in total ALP activity is observed during the first 6 months of pregnancy, followed by a rapid increase in the last trimester. This increase is primarily due to placental enzyme, but bone ALP has been reported to increase during pregnancy as well (236).

In healthy adults, the ratio of bone to liver activity is approximately 1:1. Total ALP activity is greater in men than in women between the ages of 20 and 50. The difference was thought to be due to the bone fraction, as assessed by wheat germ lectin, heat inactivation, and electrophoresis (214, 232, 237, 238); however, assessment with the Tandem-R-Ostase kit found the bone isoform to be equivalent in men and women (226). In both sexes over the age of 50, total ALP increases (237, 238). Bone ALP activity is generally found to

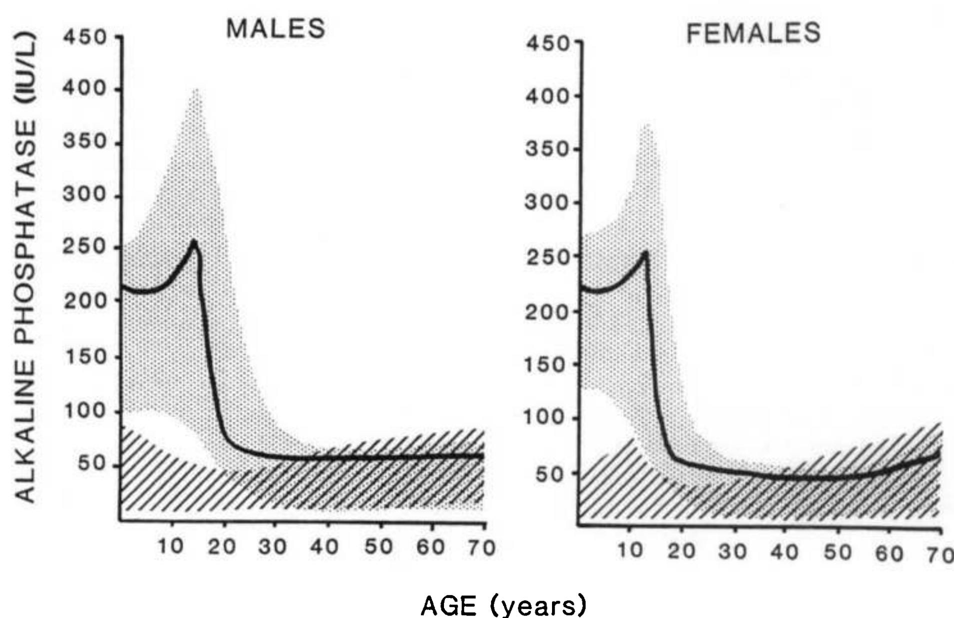

FIG. 5. Serum ALP activity in normal males and females as a function of age. The *solid line* is the mean value while the *stippled area* represents the 5% and 95% reference limits for the bone isoform. The *slashed lines* are the 5% and 95% reference limits for the liver isoform. Before puberty the bone isoenzyme represents 77–87% of the total. Activity increases in children around the age of puberty, the maximum being earlier in girls than in boys, and corresponds temporally with growth spurts in both sexes. In healthy adults, the ratio of bone and liver activity is about 1:1.

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be higher in postmenopausal women than in premenopausal women (60, 226, 237)(Fig. 5).

There is a great deal of interindividual variation in ALP levels, but for any one individual, values change little with time. ALP is cleared from the blood very slowly; the half-life varies from 40 h for bone to 7 days for placental isoforms (187). Biological daily variation of total ALP is estimated to be less than 4%. However, recent studies suggest that a slight diurnal variation in ALP activity exists that may derive from a circadian rhythm in the bone isoform (239).

Several books and comprehensive reviews have summarized changes in total and fractionated ALP in subjects with metabolic bone disease. These have focused on traditional methods for the identification of increased bone activity (187, 190, 240). In general, early studies demonstrated increased total ALP activity in patients with disorders characterized by high bone turnover. When bone formation rates are assessed by histomorphometry or isotopic calcium kinetics, weak correlations are found with total ALP (240). Total ALP has been useful when monitoring Paget's disease because the majority of activity is derived from bone (241). An elevated ALP is often the hallmark of defective mineralization observed in children with various forms of rickets or in adults with osteomalacia (242). However, in adults, because gastrointestinal and liver disorders are often the causes of osteomalacia, liver as well as bone ALP is also often elevated. In asymptomatic primary hyperparathyroidism, bone ALP may be elevated (243). In renal failure, increased ALP may be found with progressive bone disease (244, 245).

Several recent studies have quantified bone-specific ALP using the newer methods. Increased activity is found in patients with high bone turnover disorders when wheat germ lectin precipitation is used (215, 216, 246, 247). This method effectively predicts bone mineralization rates as determined by ^{47}Ca kinetics (60). Older methods (electrophoresis, heat inactivation, or chemical inhibition) show small increases (20%) in bone ALP activity in patients with osteoporosis (248). The interpretation of small increases is difficult when evaluating patients for osteoporosis because this enzyme may be elevated in disorders of mineralization such as renal osteodystrophy or osteomalacia. Using the newer methods, substantial increases have been observed in bone ALP with age and menopause in normal women. Eastell *et al.* (57, 60), using the wheat-germ lectin, reported a 73% increase in bone ALP in older women compared with women in their third and fourth decade of life. Another study using Tandem-R-Ostase found bone ALP to be increased by 77% in women within 10 yr of menopause (226). Modern assay methods should provide a substantial body of evidence in the near future regarding the utility of bone ALP in osteoporosis and other metabolic bone diseases.

B. Serum osteocalcin

Osteocalcin is one of the most extensively studied biological markers of bone formation. It is a small protein synthesized by mature osteoblasts, odontoblasts, and hypertrophic chondrocytes. While osteocalcin is primarily deposited in the extracellular matrix of bone, a small amount enters the blood. A large body of evidence indicates that serum osteocalcin is a sensitive and specific marker of osteoblastic activity, and its serum level reflects the rate of bone formation. There has been an enormous increase in the use of osteocalcin as a marker of metabolic bone disease, and serum osteocalcin levels are an accepted index of a variety of physiological states and metabolic bone disorders.

1. Biochemistry. Osteocalcin is one of the most abundant noncollagenous proteins found in bone. It is a small protein of 49 amino acids and in most species contains three residues (at 17, 21, and 24) of γ-carboxyglutamic acid (Gla), a calcium-binding amino acid (249). This vitamin K-dependent posttranslational modification of newly synthesized proteins results in γ-carboxylation of specific glutamate residues (Fig. 6). The reaction is comparable to the activation of vitamin K-dependent blood coagulation factors and is inhibited by warfarin. Several nonhuman species have one hydroxyproline residue in the primary sequence, and in humans, osteocalcin is only partially carboxylated at residue 17 (250). Two major structural features of osteocalcin are 1) the "Gla helix," a compact Ca^{2+}-dependent α-helical conformation in which the Gla residues project the Ca-binding sites into the same plane, thereby facilitating adsorption to hydroxyapatite; and 2) the COOH-terminal β-sheet, a locus for potential interaction with cellular receptors and extracellular proteins (251).

The human osteoblast produces an 11-kDa molecule consisting of a 23-residue hydrophobic signal peptide, a 26-residue propeptide, and the 49-residue mature protein (252). The pro-region contains a γ-carboxylation recognition site homologous to corresponding regions in the vitamin K-dependent clotting factors (253). After the hydrophobic region is cleaved by a signal peptidase, pro-osteocalcin is γ-carboxylated. Subsequently, the propeptide is removed and the mature protein is secreted (254) (Fig. 7).

The human osteocalcin gene is a single-copy gene located at the distal long arm of chromosome 1 (255). Multiple copies of the gene, however, exist in rat and mouse (256, 257). Various promoter elements contribute to basal expression and osteoblast specificity. The gene is further modulated by vitamin D and glucocorticoid response elements (258–261).

2. Function. The function of osteocalcin has not been defined, but its chemical structure indicates interaction with hydroxyapatite. Early studies in embryonic bone demonstrated that osteocalcin first appeared coincident with the onset of mineralization. Furthermore, increases in the protein occurred in concert with hydroxyapatite deposition during skeletal growth (262–264). It is less abundant in woven bone than in

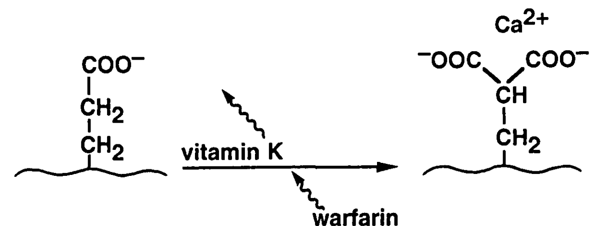

FIG. 6. Biosynthesis of γ-carboxyglutamic acid (Gla). Vitamin K and CO_2 are required for the carboxylation of specific glutamic acid residues (17, 21, and 24) in osteocalcin (17 is only partially carboxylated in humans). This reaction is inhibited by warfarin. The adjacent carboxyl groups of Gla are binding sites for Ca^{2+}.

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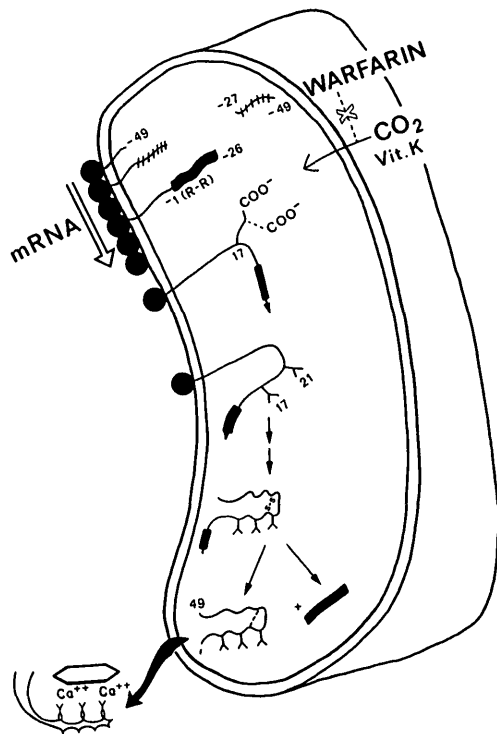

FIG. 7. Biosynthesis of osteocalcin. Pre-pro-osteocalcin consists of a 23-residue hydrophobic peptide, a 26-residue propeptide, and the 49-residue mature protein. After cleavage by the signal peptidase, the resulting proosteocalcin is targeted for carboxylation by its 26-residue propeptide. Gla (symbolized by "Y") is normally formed at residues 17, 21, and 24. After carboxylation, the propeptide is removed and the mature protein is secreted. In the presence of warfarin, partial or no carboxylation results in defective osteocalcin.

lamellar bone (265). *In vitro* studies demonstrate that osteocalcin is a marker of late osteoblast differentiation. Osteocalcin synthesis increases with mineralization and with progressive osteoblastic differentiation (205, 266, 267). Chick osteoblasts produce minimal levels of ALP and osteocalcin until they reach confluence and accumulate a dense collagenous extracellular matrix (268, 269). Coincident with the onset of mineralization of this matrix, ALP activity increases followed by induction of osteocalcin synthesis. ALP activity decreases rapidly after mineralization, but osteocalcin remains high throughout the life of the culture (270, 271). These studies suggest that osteocalcin may either limit mineralization or participate in regulation of bone turnover.

Other *in vivo* and *in vitro* studies have suggested that osteocalcin is involved in recruitment and activation of bone-resorbing cells or chemotaxis (272). Subcutaneously implanted osteocalcin-deficient bone particles show a decrease in progenitor cell recruitment, a decrease in multinucleated osteoclast-like cells surrounding bone particles, and a decrease in TRAP activity compared with normal bone (273, 274). Although others were unable to reproduce these findings, there were large inflammatory responses that may have obscured the more specific osteoclastic response (275).

Recently, a transgenic mouse has been developed in which the osteocalcin gene has been "knocked-out." The phenotype of these mice is characterized by a progressive increase in bone mass leading to bone of better biomechanical quality. Histological and histomorphometric studies performed before and after ovariectomy showed that this phenotype is due to an increase in osteoblast function without an increase in osteoblast number. Bone mineralization and bone resorption were not affected by this mutation (276).

3. Origin of osteocalcin in the circulation. Early studies suggested that circulating osteocalcin originated from bone cells and not from breakdown of bone matrix (277). Furthermore, several studies have established that serum osteocalcin is highly correlated with bone formation but not resorption, as determined by histomorphometry or calcium kinetics (43, 278–281). However, these studies did not determine whether serum osteocalcin specifically reflects matrix synthesis or mineralization, a precise distinction that is difficult histologically (282).

The *in vitro* studies cited above suggest that osteocalcin synthesis is associated with matrix mineralization rather than matrix synthesis. *In vivo* studies, however, are conflicting. Growth and mineralization of deer antler occur in two stages, providing a useful model to distinguish between matrix synthesis and mineralization. During antler growth (soft antlers), serum ALP and urinary hydroxyproline levels increase, but osteocalcin is low. During hardening of the antlers (mineralization), serum osteocalcin and 1,25-$(OH)_2D$ increase while ALP and urinary hydroxyproline levels decline (283). These data provide evidence that osteocalcin is synthesized and secreted during deposition and maintenance of mineral rather than in relation to matrix synthesis *per se*. In contrast, a study of osteomalacic subjects reported osteocalcin to be correlated with osteoid volume (*i.e.* unmineralized matrix) but not mineralization rate as determined by tetracycline labeling (284). Yet others have demonstrated a very high correlation between osteocalcin and mineralization rate determined by calcium kinetics in osteomalacic subjects (r = 0.92)(285). It is difficult to draw any conclusions from studies of patients with osteomalacia, because such an abnormal state is likely to confound regulation of osteocalcin biosynthesis, secretion, or clearance. Indeed, in one study in which rats were made osteomalacic by calcium, phosphate, or vitamin D deprivation, the osteocalcin response varied with the nature of dietary restriction, yet all animals had clearly undermineralized bones (286).

Because matrix synthesis and mineralization are linked in most clinical situations, it is appropriate to interpret osteocalcin data in terms of changes in bone formation. In fact, in most clinical situations (osteoporosis being the most notable exception), bone formation and resorption are tightly coupled. Therefore, serum osteocalcin should be regarded as a measure of bone formation in particular and bone turnover in general.

4. Methodology. The first osteocalcin assays were competitive RIAs using bovine osteocalcin, purified to homogeneity, as the antigen. Antibody to bovine osteocalcin cross-reacts with human osteocalcin, and purification of bovine osteocalcin is more convenient and gives higher yields of protein (287–291). For these reasons, most first generation assays used bovine-directed antibody. Yet considerable inconsistency is evident when comparing values from various laboratories

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using a variety of osteocalcin RIAs. Wide variations are reported in control and patient populations. Although some discrepancies can be attributed to heterogeneous sample populations, analytical differences also account for some of this variability. Chemical reagents and methodology may contribute to differences among assays, but antibodies and standards are the most likely source of variability. One report, in which an osteocalcin standard and 10 unknown serum samples were provided to eight different laboratories *each with their own in-house osteocalcin assays*, clear differences in absolute values of the shared samples were found. Differences persisted even when the provided standard was used to calibrate the individual assays, suggesting that the major variability resided in the various antibodies. Nevertheless, the various assays gave reasonably consistent values in a number of metabolic bone diseases when the results were expressed as a percent of the laboratory's normal value (292). In a second study, on the other hand, eight *commercially available kits* were used to determine osteocalcin levels in nine healthy controls and 38 individuals with conditions known to affect bone turnover. The results were discordant even when they were normalized to healthy control values (Z scores). Furthermore, recovery of added osteocalcin varied widely (293).

In another study, four different antibodies were used to measure osteocalcin in serum and bone. There were no differences in the amount of osteocalcin detected in the bone, but the amount in the serum varied (294). These data suggest that various forms of osteocalcin circulate and that individual antibodies recognize them differently. There is a great deal of interest in identifying the nature and source of this circulating heterogeneity. We have identified fragments of osteocalcin from serum of patients with end-stage renal disease and have suggested that limited proteolysis of osteocalcin occurs in extrarenal sites (295). Other laboratories have verified this circulating heterogeneity of osteocalcin in other disease states (Paget's disease) and in normal individuals (294, 296–299). It is clear that different antibodies detect different subforms or fragments of osteocalcin. In some assays, epitope specificity and the degree of reactivity with multiple circulating forms of the protein are unknown. In a recent report, all tested commercial kits that employed a single antibody RIA could recognize, to varying degrees, cathepsin-derived fragments of osteocalcin (300). This variability among assays complicates the clinical interpretation of osteocalcin results in patients with metabolic bone disease.

In an attempt to clarify this issue, several laboratories have developed two-site immunoassays that recognize only the intact molecule (301–307). Under controlled circumstances, these correlated well with conventional "in-house" RIAs. However, the first and last amino acids must be recognized by the antibodies, or large fragments will be detected. This is illustrated by the fact that one laboratory developed two separate assays for intact osteocalcin both in two-site formats. The same capture antibody was used in both assays but two different monoclonal antibodies, both directed to the same 12-amino acid sequence, were employed for read-out. These assays gave correlated but different absolute values with the same serum samples (and using the same standards) (307). Even assays claiming to be similar are not interchangeable, and the precise molecular structure being detected should be clearly defined when osteocalcin assays are employed.

Hemolysis and lipemia will confound assay results. Proteases released by red cells degrade the protein, resulting in reduced values (308). Osteocalcin may bind to lipid, rendering it nonimmunoreactive. Freeze-thawing considerably reduces values, and a decrease in concentration is often found by the second or third freeze-thaw cycle (300, 308). Presumably, osteocalcin is degraded by serum proteases during sample handling, because purified osteocalcin is stable to repeated freeze-thaw cycles. (C. Gundberg, unpublished observations). With some assays, differences are also observed with serum or plasma (308, 309).

A new generation of assays is based upon the observation that the major circulating forms of the protein appear to be the intact and the large N-terminal fragment spanning residues 1–43 (297, 310). Whether this major fragment is derived from osteoblastic synthesis, catabolism of the intact protein in bone, or by proteolysis in blood or other tissues is not firmly established (294, 295, 297, 299, 311). Furthermore, it is not known whether variable circulating fragments of osteocalcin occur in disease states. Nevertheless, several studies have shown that these assays are robust and are specific for bone formation. There are numerous commercial kits that measure osteocalcin. These are listed in Table 4 along with their specifications.

5. Clinical correlates. Serum osteocalcin is greater in infants and children than in adults, with peak values occurring at puberty. The changes in serum osteocalcin are more strongly correlated with growth velocity than chronological age and parallel related changes in ALP, urinary hydroxyproline, and the N-telopeptide of collagen (130, 312–315). In adults, osteocalcin levels are relatively stable but start to rise in men after the age of 60 (316–324)(Fig. 8). Several studies in women show a rise with menopause which is correlated with an increase in bone turnover rate as assessed by histomorphometry and calcium kinetics (278–280). Racial differences have also been identified. Bell *et al.* (325) reported lower serum osteocalcin in African-American adults compared with Caucasians; and Villa *et al.* (326) found that levels in Mexican-American subjects were higher. We found higher osteocalcin levels in Mexican-Americans than in non-Hispanic whites, but no differences between (non-Hispanic) whites and blacks in a large sample of 3016 men and women who participated in the third National Health and Nutrition Examination Survey (327).

Osteocalcin is a relatively small protein that is rapidly filtered by the kidney and degraded (277, 328). Serum levels are reported affected by changes in renal function. When renal glomerular function is impaired, circulating osteocalcin increases (329). This occurs when glomerular filtration rate is below 20–30 ml/min per 1.73 m^2 body surface area, or serum creatinine is greater than 160 μmol/liter (330). In children, increases occur at glomerular filtration rate below 40 ml/min per 1.73 m^2 (331). With advanced renal failure, serum osteocalcin is invariably elevated, ranging from 2–200 times higher than normal, and correlated with serum creatinine (332–336).

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TABLE 4. Commercially available assays for formative markers of bone turnover

Assay name	Manufacturer	Analyte	Assay method	Sample requirements[a]	Sensitivity
Iso-ALP	Boehringer Mannheim, Mannheim, Germany	Bone-specific ALP	Wheat-germ-lectin precipitation followed by colorimetric method	Serum; volume dependent upon colorimetric method	Dependent upon colorimetric method
Tandem-R Ostase	Hybritech Inc., San Diego, CA	Bone Specific ALP	IRMA, double antibody	100 μl serum	2 μg/liter
Alkphase-B	Metra Biosystems, Inc., Mountain View, CA	Bone Specific ALP	ELISA, single monoclonal antibody	20 μl serum	0.7 U/liter
Bovine osteocalcin	Biomedical Technologies Inc., Stoughton, MA	Bovine osteocalcin[b]	RIA, polyclonal antibody	50 μl serum or heparinized plasma	1.0 μg/liter
Human osteocalcin	Biomedical Technologies Inc., Stoughton, MA	Human osteocalcin	RIA, polyclonal antibody	50 μl serum or heparinized plasma	1.0 μg/liter
Intact osteocalcin	Biomedical Technologies Inc., Stoughton, MA	Intact human osteocalcin	ELISA, double antibody	20 μl serum	0.5 μg/liter
Mid-Tact Osteocalcin	Biomedical Technologies Inc., Stoughton, MA	Intact and N-terminal osteocalcin fragment	ELISA, double antibody	20 μl serum	1.0 μg/liter
Ostk-PR	CIS-bio International, Gif-sur-Yvette, France	Bovine osteocalcin[b]	RIA, polyclonal antibody	100 μl serum or heparinized plasma	1 μg/liter
ELSA-OST-NAT	CIS-bio International, Gif-sur-Yvette, France	Intact human osteocalcin	IRMA, double antibody	50 μl serum	0.3 μg/liter
ELSA-OSTEO	CIS-bio International Gif-sur-Yvette, France; and CIS-US, Inc., Bedford, MA	Intact human osteocalcin and N-terminal mid molecule fragment	IRMA, double antibody	50 μl serum	0.4 μg/liter
Osteocalcin	Diagnostic Systems Laboratories, Webster, TX	Human osteocalcin	RIA, polyclonal antibody	50 μl serum	0.4 μg/liter
OSCAtest osteocalcin	Henning, Berlin, Germany	Human osteocalcin	RIA, polyclonal antibody	50 μl serum	0.5 μg/liter
Osteocalcin	Incstar Corp., Stillwater, MN	Bovine osteocalcin[b]	RIA, polyclonal antibody	50 μl serum or heparinized plasma	0.4 μg/liter
NovoCalcin	Metra Biosystems, Inc., Mountain View, CA	Bovine osteocalcin[b]	ELISA, single monoclonal antibody	25 μl serum	0.45 μg/liter
Human osteocalcin	Nichols Institute	Intact human osteocalcin	IRMA, double antibody	25 μl serum	0.1 μg/liter
Procollagen PICP	Incstar Corp., Stillwater, MN; and Orion Diagnostica, Espoo, Finland	PICP	RIA	100 μl serum	0.2 μg/liter
Prolagen-C	Metra Biosystems, Inc., Palo Alto, CA	PICP	ELISA, double antibody	100 μl serum	1.0 μg/liter

[a] Sample Requirements are per tube or well; assays require samples to be run in duplicate or triplicate.
[b] Cross reacts with human osteocalcin.

Osteocalcin levels also follow a circadian rhythm characterized by a decline during the morning to a noontime low followed by a gradual rise that peaks after midnight. Observed differences between the peak and nadir range from 10–30% (337–340). Serum osteocalcin levels have been reported to vary during the menstrual cycle, with the highest levels observed during the luteal phase. During pregnancy, osteocalcin levels decrease throughout the first and second trimester but return to normal just before delivery (341–344).

Changes in serum osteocalcin in individuals with various bone disorders are summarized in several comprehensive

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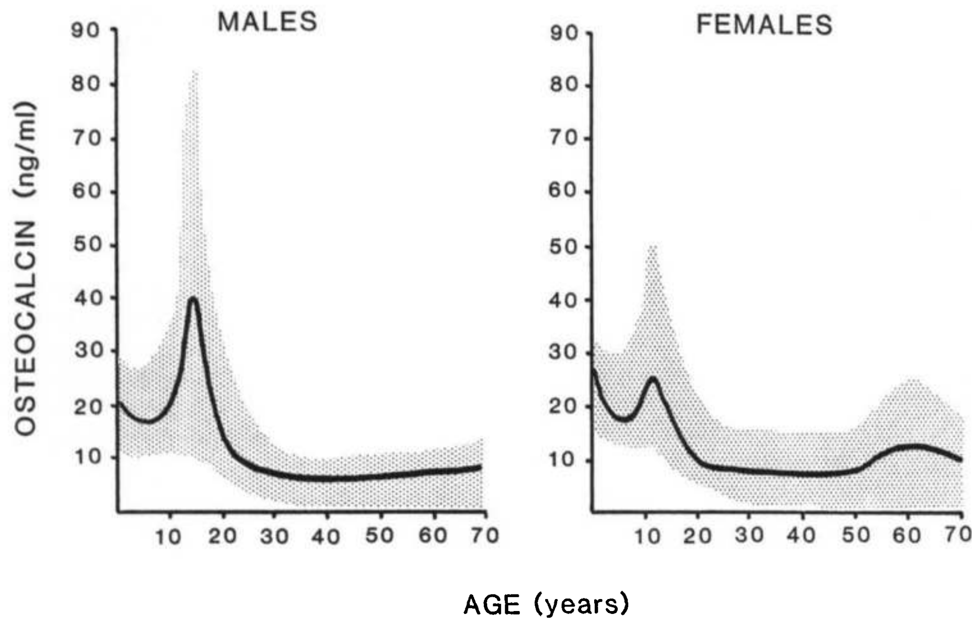

FIG. 8. Serum osteocalcin concentrations in normal males and females as a function of age. The *solid line* is the mean value while the *stippled area* represents the 5% and 95% reference limits. Serum osteocalcin is higher in children than in adults. Peak values occur during the pubertal growth spurt. In adults, osteocalcin levels are relatively stable but start to rise in men after the age of 60. Elevations are also noted in women after the menopause.

reviews (261, 345, 346). In general, serum levels of osteocalcin are elevated in patients with diseases characterized by high bone turnover rate and reflect the expected changes in bone formation after surgical or therapeutic intervention. An exception is found in Paget's disease in which serum ALP (either total or bone specific) is a better predictor of severity of disease than osteocalcin (347). This discrepancy may reflect the greater abundance of osteocalcin in woven bone compared with lamellar bone (265). Osteocalcin levels are not affected by illnesses that do not involve bone (348). In certain circumstances, however, other determinants can influence circulating osteocalcin concentrations. Drug-induced alterations in normal hydroxyapatite-protein interactions may affect the apparent circulating concentrations of osteocalcin. Coumarin anticoagulants, which interfere with normal vitamin K-dependent carboxylation, will reduce binding of osteocalcin to bone hydroxyapatite, resulting in a greater proportion of newly synthesized, but undercarboxylated, osteocalcin in the circulation (349, 350).

In postmenopausal osteoporotic women, serum levels of osteocalcin have been reported to be normal, low, or high (51, 278, 279, 351–356). This varied response has been attributed to the variability in bone formation rate observed in this condition. The usefulness of serum osteocalcin as an indicator of bone turnover rate in women with osteoporosis has been validated by a significant correlation between serum levels and histomorphometric indices of bone formation. The increased fracture risk after menopause is reflected by the coincident rise in mean serum osteocalcin in normal women, which is itself inversely correlated with changes in bone mineral density at the lumbar spine, midradius, and distal radius (352, 355, 356). In a study of 110 normal postmenopausal women Yasumura *et al.* found that total body calcium and phosphorous, determined by total body neutron activation, was lower in those women with elevated osteocalcin levels than in those with normal levels, indicating the relatively reduced bone mineral content of a high turnover state (357). In groups of subjects, serial measurement of osteocalcin has proven useful in monitoring the response to treatment of osteoporosis (70, 358–360). Estrogen therapy decreases serum osteocalcin levels (361), and stimulation of bone formation with slow-release sodium fluoride is accompanied by an increase in serum osteocalcin (362).

It has been assumed that vitamin K deficiency is rare because of both the widespread distribution of phylloquinone (vitamin K_1) in green vegetables and also the bacterial synthesis of menaquinone (vitamin K_2). However, vitamin K sufficiency has traditionally been assessed only by functional one-stage prothrombin times, which do not consider nonliver vitamin K requirements. Studies indicate that bone, however, is more susceptible to vitamin K deficiency than is liver (363). Assessment of undercarboxylated osteocalcin is potentially useful for determining *subtle* changes in vitamin K status. This measurement has been developed with the recognition that affinity of osteocalcin for hydroxyapatite depends on the number of Gla residues in the protein (277). In a recent paper, Sokoll *et al.* (350) showed that a low dose of warfarin resulted in a 2.7-fold increase in undercarboxylated osteocalcin with no effect on prothrombin times. Osteocalcin was normally carboxylated within 2 days of vitamin K treatment. Total osteocalcin (carboxylated plus undercarboxylated) was unaffected by either treatment, indicating that synthesis of the protein did not change.

Interestingly, in one study undercarboxylated osteocalcin was higher in postmenopausal women than in premenopausal women. When vitamin K supplements were given to the postmenopausal women, normal carboxylation levels were restored (364). This finding has raised the possibility

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that vitamin K deficiency, induced by either low dietary intake or by coumarin anticoagulants, may have an adverse effect on bone. In subsequent studies, plasma vitamin K levels in elderly osteoporotic patients with fractures of the femoral neck were reduced compared with aged matched controls (365). Furthermore, in elderly institutionalized women, increased undercarboxylated osteocalcin was predictive of subsequent hip fracture (366). Certainly, a variety of nutritional factors, including vitamin K, may contribute to poor skeletal health. Whether poor vitamin K status in these subjects merely reflects general poor nutrition is unknown. However, when a subclinical vitamin K deficiency was induced by a vitamin K-deficient diet in normal adults, younger subjects were more susceptible to an acute deficiency than were elderly individuals (367), and plasma phylloquinone levels were lowest in women in their twenties (368). If, in fact, vitamin K deficiency occurs in the elderly, then depletion of body stores requires a much longer period of deprivation than in the younger population.

Several authors have suggested that vitamin K deficiency or coumadin therapy reduces the content of osteocalcin in bone and that this has an adverse effect on bone. However, there is no consistent effect of long-term coumadin on bone density (369–371). The fact that the osteocalcin "knock-out" mice do not have either reduced bone density or increased fracture rates obviates a direct role for osteocalcin in any potential negative effects of vitamin K deficiency on bone. However, other vitamin K-dependent proteins in bone and other tissues involved in calcium homeostasis may be involved in the observed bone pathology (249). Nevertheless, the estimation of the degree of osteocalcin carboxylation may prove to be a useful measure of vitamin K status.

C. Serum type I procollagen peptide

For many years, there has been an interest in developing reliable markers for bone collagen synthesis. The procollagen extension peptides, which guide assembly of the triple helix, are cleaved from the newly formed molecule in a stoichiometric relationship with collagen biosynthesis. This should reflect bone formation in a manner analogous to the assessment of C peptide for endogenous insulin production.

1. Biochemistry. As outlined above, during collagen synthesis, intramolecular disulfide bonds form between the three carboxyl propeptides and guide helical formation. Specific endopeptidases cleave the procollagen molecule at precise sites in each chain, first at the amino terminus and then at the carboxyl terminus. Type I collagen propeptides are produced not only by bone but by other tissues that synthesize type I collagen: skin, gingiva, heart valve, dentin, cornea, fibrocartilage, and tendon.

Procollagen type I carboxy-terminal propeptide (PICP), with a molecular mass of 117 kDa, is a trimeric globular glycoprotein with asparagine-linked carbohydrate units (372). PICP, stabilized by disulfide bonds, circulates as a single molecule. It has a serum half-life of 6–8 min, being cleared in the liver endothelial cells by the mannose receptor (373).

The procollagen type I amino-terminal propeptide (PINP), a 70-kDa globular protein, contains an internal region of 17 contiguous GLY-X-Y triplets containing proline and hydroxyproline in the same proportion as the collagen molecule (374). PINP can be cleared from the circulation by the scavenger receptor of liver endothelial cells. Some of the large nondialyzable hydroxyproline peptides found in urine are derived from cleaved free PINP (375, 376). In addition, PINP can be deposited directly into bone similar to the deposition of amino-terminal peptide of collagen type III in the skin matrix. Fisher *et al.* (377) have isolated a 24-kDa phosphoprotein from bone that was shown to have the identical amino acid sequence as the amino terminus of the $\alpha1$(I) chain of type I procollagen. This material constitutes 5% of the noncollagenous protein in bone. The compact helical nature of the phosphorylated peptide allows it to interact with mature collagen fibrils and be sequestered into bone. Some PINP may be deposited directly into bone after extracellular cleavage from the triple-helical collagen molecule or it may enter the circulation and later become entrapped in the matrix.

2. Methods. Four assays have been described for measurement of PICP (378–381). Three are based on polyclonal antiserum made against purified collagen isolated from human skin or lung fibroblast cultures. The collagen is treated with bacterial collagenase to liberate the propeptide from the mature molecule. Immunogen, standards, and tracer were all derived from the same material. Most of the available clinical data have been obtained with one of these assays used by the developers (378) and by others using commercial forms of the assay (PICP, Orion Diagnostics/US-INCstar, Stillwater, MN; and Metra BioSystems, Mountain View, CA). All data demonstrate that PICP is stable during storage at −20 C for several months and after repeated freezing and thawing (378, 381).

The use of bacterial collagenase to cleave the propeptide from collagen results in an amino terminus that differs from that produced *in vivo* by specific human endopeptidases. A fourth assay uses purified *free* procollagen peptide from human fetal fibroblasts as standards and tracer (381). Antiserum was made against partially purified PICP from human amniotic fluid. The correlation (*r*) between these two approaches was 0.98, but the normal values differed by 25%. This may be due to immunological variability or differences in calibration.

Assays have also been developed to measure PINP in blood. Ebeling *et al.* (382) used a synthetic amino-terminal sequence spanning residues 7–24 of the $\alpha1$- chain of PINP to immunize rabbits and for standards and tracer. Competitive binding curves for purified PINP and human serum samples indicated immunological identity between samples and standard. However, when PINP and PICP concentrations were determined in the same set of patient samples, there was no correlation between the two, and PINP concentrations were 100 times higher than those of PICP.

A second assay for PINP used the 23–34 sequence for assay development and reagents (383). In contrast to the above, PICP and PINP levels in adults were of the same order of magnitude but did not correlate. Conversely, in children PINP concentrations were 2–3 times greater than PICP, but

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the two correlated well, suggesting differences in metabolic clearance of the two peptides in adults and children.

Recently, an immunoassay for the intact, trimeric form of circulating PINP has been described (384). Studies with this assay may clarify some of the discrepancies observed with the peptide-based assays.

3. *Clinical correlates.* There is considerable sequence and structural homology between PICP and PIIICP, the latter being derived from type III collagen in soft tissues. Simon *et al.* (379) found evidence of as much as 20% cross-reactivity between PICP and PIIICP using one of the earliest PICP RIAs. However, because the serum concentration of PIIICP is 1 order of magnitude lower than that of PICP, the effects on measured PICP concentration should be small.

None of the assays eliminate the potential contribution to circulating PICP from soft tissue synthesis of type I collagen. The rate of turnover of collagen in bone is faster than in other tissues, and, therefore, changes in PICP are assumed to reflect changes primarily in bone collagen synthesis. Studies in pigs have shown that the contribution to the serum pool of PICP by lymph draining skin and other tissues is small (385). However, a histomorphometric study by Parfitt *et al.* (386) suggested that some of the PICP in blood must be derived from biosynthesis in soft tissues. Furthermore, Christiansen and co-workers (387–389) found both PICP and PIIICP to be increased in patients given nandrolone decanoate, but no changes in histomorphometric measurements of bone formation, in osteocalcin, or ALP were observed. Therefore, the degree of specificity of PICP for bone formation is yet to be resolved.

PICP follows a circadian rhythm similar to the other bone markers, with peak values occurring in the early morning hours (~0300 h) and nadirs in the afternoon (122, 390). In one study in which blood was collected every 15 min for 4, 6, or 8 h, there were marked pulsatile fluctuations in PICP that varied up to 4-fold, suggesting a larger intraindividual variation than is usually observed for formative markers (391).

Serum concentrations of PICP are elevated in disorders characterized by high bone turnover such as hyperparathyroidism, hyperthyroidism, osteomalacia, Paget's disease, and cancer patients with osteoblastic metastases (123, 285, 392–395). PICP levels related to histomorphometric and calcium kinetic measures of bone formation in patients with both high and low turnover bone disease (123, 396). In Paget's disease, values decline after treatment with calcitonin or bisphosphonates (380). In osteogenesis imperfecta type IA, a disease characterized by defective collagen synthesis, serum PICP concentrations were reduced and were positively correlated with bone mineral density (397).

As with the other bone markers, PICP concentrations are generally related to linear growth in children (390, 398, 399). PICP values decline with age in men but increase in women (378, 382). Two studies found a slight but insignificant increase in PICP after the menopause (391, 400), but another study found no change (381). In patients with osteoporosis there was a modest correlation ($r = 0.4$) with histomorphometric indices of bone formation, but it did not correlate with spinal bone mineral density as do other measures of bone turnover (401). It could, however, predict differences in lumbar spine density in dizygotic twins (402).

The degradation of PICP is primarily via hepatic pathways and, as a consequence, PICP levels are elevated in patients with liver disease (373, 386, 403). On the other hand, because of its large molecular weight, PICP is not filtered by the kidneys, and serum levels of PICP are not dependent upon renal function. As a consequence, PICP could be a useful index of bone turnover in renal disease. Several studies have shown that serum PICP levels are significantly correlated with histomorphometric indices of bone formation in patients with chronic renal failure (404–406). This distinguishes it from osteocalcin, which is cleared by the kidney and whose concentration in renal disease is dependent both on renal clearance and bone turnover. Furthermore, one study found that PICP levels were not different in renal patients with and without liver disease (404), making its measurement superior, in this instance, to ALP as well. However, PICP concentrations are inappropriately elevated in patients on hemodialysis with significant aluminum overload (407). The potential use of this assay will depend on developing a method of correcting for differences in metabolic clearance by the liver and on ascertaining the contribution from tissue sources other than bone.

IV. Comparative Studies

From the above review of serum and urine indices of bone turnover, it is clear that most studies have focused on individual markers in normal and diseased states, and several reviews have discussed the clinical relevance of the bone markers (408–416). A few studies have used several markers to verify changes in bone turnover in clinical studies. With the advent of several new markers in the past 5 years, the persistent question has been: which marker gives the most reliable information in the evaluation of metabolic bone disease? A few studies have addressed this question by comparing several different biochemical markers in particular disease states with direct measures of bone status or activity such as calcium kinetics, histomorphometry, or bone density. Here we will review these various studies.

A. Resorptive OR formative studies

1. *Resorptive.* The resorptive markers hydroxyproline, GHYL, Pyr, and Dpy (all measured by HPLC) have been compared. The results showed that all had comparable discriminating power for patients with high bone resorption (Paget's disease and hyperparathyroidism) but only GHYL and the pyridinoline cross-links performed well for postmenopausal women with mildly elevated bone resorption (86). Fledelius *et al.* (417) found free Pyr by ELISA to be less sensitive than Dpy or total pyridinolines by HPLC in a study of postmenopausal women on hormone replacement therapy.

Blumsohn *et al.* (145) compared a full range of resorption markers in 14 patients with Paget's disease being treated with etidronate. In urine, the assays were total and free pyridinolines measured by HPLC and ELISA, and NTx and CTx measured by ELISA and hydroxyproline. In serum, TRAP and ICTP were measured; however, the TRAP assay

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employed was not for the bone-specific isoenzyme. The greatest suppression from baseline urine levels on therapy was seen with the telopeptide assays, NTx and CTx (75% suppression at 6 months). Next were total pyridinolines by HPLC (45–60% decrease), hydroxyproline (40%), and then free pyridinolines by ELISA (10–40%). ICTP and TRAP showed minimal response (145).

2. *Formative.* In studies in which only the formative markers were compared, PICP was generally inferior to osteocalcin and bone-specific ALP. Ebeling *et al.* (382) measured PICP by a commercial kit and osteocalcin and ALP with "in-house" assays in 169 normal subjects and 197 patients with various metabolic bone diseases. In the patient samples, differences from normal were less pronounced for PICP than with either osteocalcin or bone-specific ALP. The same observation was made by Minisola *et al.* (392) in patients with primary hyperparathyroidism, before and after surgery. Likewise, when compared with calcium kinetic data, osteocalcin was found to be a reliable marker of mineralization rate in all diseases studied, whereas total ALP and PICP showed disease-specific discrepancies (396).

B. Simultaneous resorptive and formative studies

Multiple markers of both formation and resorption have been used both to validate the markers and to understand changes in bone physiology that occur during growth, lactation, and menopause as well as in disease. The main interest, however, is in the ability of bone markers to predict bone loss in osteoporosis and to monitor response to therapies.

1. *Normal growth and development.* Blumsohn *et al.* (83) measured total and bone-specific ALP by wheat germ lectin precipitation; osteocalcin by a homologous RIA; PICP, ICTP, immunoreactive total pyridinolines, Dpy, and GHYL by HPLC; and TRAP by (non-bone-specific) kinetic assay in 91 healthy girls during puberty. All markers increased maximally during midpuberty (Tanner stages II and III) and decreased toward adult levels in late puberty. The parallel increase in both formative and resorptive markers no doubt reflects growth, modeling, and remodeling of bone tissue that accompanies skeletal growth. Relatively greater increases, however, were found for bone-specific ALP, osteocalcin, and urinary Dpy than for all other markers, suggesting greater sensitivity of these markers to changes in bone turnover during pubertal growth.

Likewise, Sowers *et al.* (154) assessed bone loss and recovery in 115 lactating and nonlactating postpartum women. Osteocalcin (by heterologous RIA), bone-specific ALP (by chemical inhibition), and N-telopeptide (by ELISA) were all significantly increased in lactating women compared with nonlactating women (no other markers were measured). All three markers increased during breast feeding and declined when lactation stopped and were equally correlated with changes in bone mineral density at the lumbar spine and femoral neck ($r = -0.49$ to -0.53). The return to menses and ovarian stimulation, however, resulted in a more rapid decline in the N-telopeptide than either of the two formation markers. This could explain the recovery of bone mass after lactation ceases. This study clearly illustrates the potential of bone markers to reveal mechanisms of bone loss and gain in different physiological states.

2. *Metabolic bone disease.* In hyperthyroidism, "total" osteocalcin (by an IRMA specific for both intact and large N-terminal fragments) and urinary free Pyr by ELISA were significantly elevated in most patients, correlated with T_3, and decreased after therapy. Bone-specific ALP (by IRMA) was elevated in only 11 of 27 patients, did not correlate with T_3, and increased transiently after therapy (147). Treatment of hyperthyroidism often results in transient increases in liver function tests, and the increase in bone-specific ALP in this study may result from the cross-reactivity with the liver isoenzyme observed with this particular assay.

Similarly, when exogenous GH was given as a stimulation test to 15 subjects with postmenopausal osteoporosis and 15 aged-matched controls, markers of bone resorption (ICTP and hydroxyproline) and formation (osteocalcin and PICP) increased, but bone-specific ALP (wheat germ lectin) decreased. The same pattern was found in both subjects and controls (418). A transient decrease in total ALP was also observed when GH was given to normal male volunteers aged 21–31 (419). In contrast, when the hormone was given to GH-deficient children and adults, all bone markers, including bone-specific ALP, increased (420, 421). These findings might be explained if GH stimulates ALP activity in osteoblasts but suppresses activity in primary hepatocytes.

3. *Osteoporosis.* It is generally accepted that the accelerated rate of bone loss that occurs after the menopause is due to increased turnover. Studies using bone-specific markers, however, have suggested that increased bone turnover is also responsible for the slow phase of bone loss in elderly women (60). In a recent study of 653 elderly women, Garnero *et al.* (422) measured intact osteocalcin, bone-specific ALP by IRMA, PICP by RIA, and NTx and CTx by ELISA. They found that menopause induced a 37–52% and 79–97% increase in bone formation and resorption marker levels, respectively (except for PICP which did not change). Furthermore, the markers remained elevated in those women with the lowest bone density up to 40 yr after the menopause.

In a clinical study designed to compare biochemical markers in late postmenopausal osteopenic women and their response to therapy, Garnero *et al.* (141) measured osteocalcin (IRMA specific for both intact and N-terminal), bone-specific ALP (IRMA), PICP, total Pyr and Dpy (HPLC), N-telopeptide (ELISA), free Pyr (ELISA), and serum ICTP (RIA) (141). All markers except PICP and ICTP were significantly increased above the premenopausal mean in these subjects; N-telopeptide was the most elevated (171% increase). In women treated with alendronate, all resorptive markers declined but only N-telopeptide and Dpy by HPLC were reduced to the premenopausal values. Formation markers fell to normal or subnormal (PICP) premenopausal values several months after the fall in the resorptive markers. The percent decrease at 3 months correlated highly with the percent increase in spinal bone mineral density at 24 months for all three formation markers but only for total Dpy by HPLC and N-telopeptide by ELISA of the resorptive markers.

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Prestwood *et al.* (146) measured osteocalcin (RIA), bone-specific ALP (IRMA), PICP, hydroxyproline (colorimetric), total pyridinolines (HPLC), total Dpy (HPLC), NTx (ELISA), free Pyr (ELISA), and serum ICTP (RIA), in a study of the effect of conjugated estrogen in 15 women over 70 yr of age without history of fracture. All resorption markers fell during hormone replacement therapy with the greatest change in the NTx and total Dpy (40%). All resorption markers rose back to baseline levels after therapy ceased with the exception of ICTP which fell 20% during therapy and remained there post therapy. All formation markers decreased during estrogen replacement therapy (osteocalcin, 32%; bone-specific ALP, 17%; and PICP, 8%). Osteocalcin returned to baseline levels 6 weeks post therapy while the other two markers remained below baseline.

In a subsequent study, Raisz *et al.* (423) examined the effects of adding a small dose of oral androgen for 9 weeks to older postmenopausal women on estrogen replacement therapy. Patients treated with estrogen alone showed a decrease in osteocalcin (homologous RIA), bone-specific ALP (IRMA), PICP, and Dpy, Pyr, and hydroxyproline (all by HPLC). Dpy exhibited the greatest decline of the resorption markers, while bone-specific ALP fell by 26%, OC by 40%, and PICP by 15%. When methyltestosterone was added, the inhibitory effects of estrogen on the resorption markers was not altered, but there was a reversal of the inhibitory effect on the formation markers. These studies suggest that a combination of estrogen and androgen may result in greater benefit to bone than when estrogen is used alone and underscore the value of using *both* resorption and formation markers.

Finally, in an evaluation of cyclical PTH and calcitonin therapy for osteoporosis, Hodsman *et al.* (70) measured osteocalcin (heterologous RIA), total ALP, PICP, hydroxyproline (colorimetric), and total Dpy (HPLC). A continuous 24-h infusion of PTH(1–34) caused osteocalcin, ALP, and PICP to fall by 39%, 49%, and 9%, respectively, while hydroxyproline and Dpy increased by 48% and 5%, respectively. With daily injection of PTH(1–34), however, all formative markers and hydroxyproline increased (from 100% to 240%) but Dpy did not change. In those subjects also receiving calcitonin, osteocalcin and hydroxyproline fell but there was no effect on the other markers. In general, of the three formation markers, osteocalcin gave the largest response to PTH and correlated best with histological indices of bone formation. There were few correlations for PICP and none for ALP. Urinary hydroxyproline, but not Dpy, correlated with surface osteoclast counts. The poor performance of Dpy in this study is surprising and it would be of interest to determine whether other methods for measuring collagen cross-links (as well as fractionating ALP) would have provided the same results. Nevertheless, this study confirms in humans what has previously been observed in animals (424–426), that the actions of PTH are anabolic when given by intermittent injection but inhibit formation if given by continuous infusion (427).

All of these studies illustrate the potential usefulness of the bone markers in clinical studies. They can provide insight into physiological processes and correlative information on new therapies for the treatment of osteoporosis. If the effects on bone markers prove to be predictive of changes in bone density or fracture incidence, then their measurement may prove to be extremely useful in clinical trials of new therapeutic agents. Nevertheless, in evaluating any study an important caveat emerges. The conclusions are highly dependent not only on the marker chosen but also on the method used for its measurement. Collagen catabolism may best be assessed by excretion of collagen cross-links, but which method and analyte provides the most specific measurement of bone resorption is yet to be resolved. Use of the NTx, CTx, ICTP or the various free pyridinolines by HPLC or ELISA may give varying degrees of precision and sensitivity. These various assays may correlate with each other to greater or lesser extent depending on the disease state, subject group, and laboratory carrying out the study. As more independent, direct comparisons are performed, the biological significance and relative specificity of these various analytes and assays to bone metabolism should become clearer.

Some of the studies summarized above reflect these difficulties. For example, HPLC requires careful standardization and quality control, which may vary from laboratory to laboratory. For osteocalcin, kit comparisons have been conducted only with the intent of determining differences or similarities between kits and not to define which method is more accurate in a specific clinical situation. This is further complicated by the fact that some kits measure only intact osteocalcin while others measure a variety of fragments which, in most cases, are undefined (428). Which form of osteocalcin is the most physiologically relevant is not established. It appears that methods to measure PICP and PINP are evolving, and future assays may resolve some of the discrepancies that have been observed with these assays. Precise understanding of the various forms of ALP in normal and disease states will allow for refinement of antibodies for bone-specific ALP and will hopefully eliminate the cross-reactivity with the liver isoform that currently exists. Finally, there is little information of the metabolic clearances of any of these analytes, and whether disease states or therapeutic agents will alter their clearance is largely unknown.

V. Practical Applications for Bone Markers

A. Research

Although we have not specifically reviewed the literature on bone markers in basic rather than clinical research, data from animal models and *in vitro* studies are available. For example, the identification of isolated cells as osteoblasts requires that the cells respond to PTH and that they produce osteocalcin. Furthermore, knowledge of the differentiation of osteoblasts has been aided by studying osteocalcin, collagen, and ALP production. In animals, bone markers are used to demonstrate the effect of a variety of factors that control bone, including weightlessness, immobilization, ovariectomy, and the actions of cytokines, hormones, and drugs. Such studies have been limited somewhat by the lack of species-specific reagents for some of the markers (*e.g.* PICP, ICTP, NTx, CTx). These assays are based on specific antibodies to the human peptides which may not always cross-react with the animal forms. However, many assays are species independent (*e.g.* bone-specific ALP determined by

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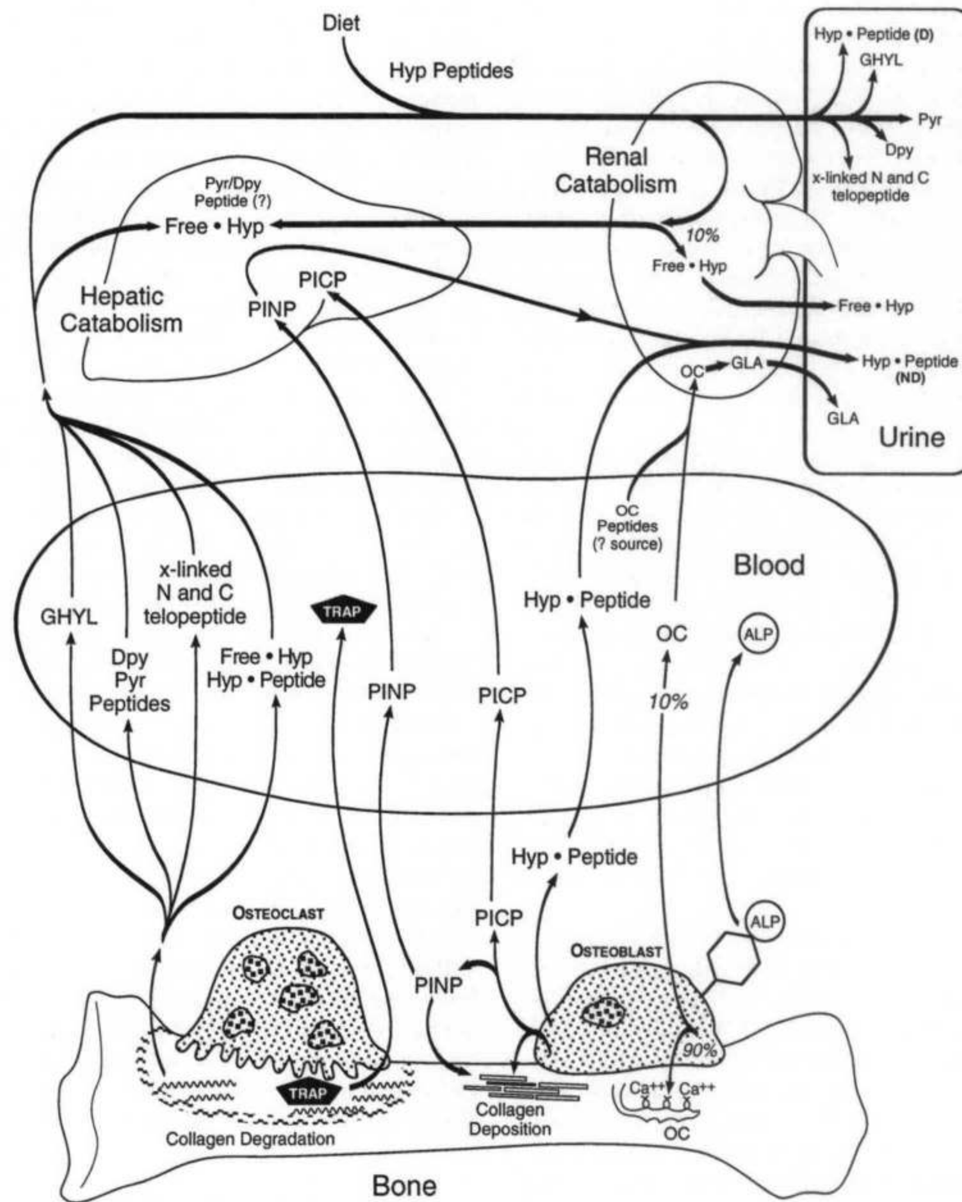

FIG. 9. Schematic representation of synthesis and catabolism of the various bone markers. *Resorptive markers*: When osteoclasts resorb bone, they secrete collagenase, which releases fragments of the collagen fibrils into the circulation. Some of these fragments may contain hydroxyproline (Hyp), galactosyl hydroxylysine (GHYL), and glucosyl galactosyl hydroxylysine (Glc.GHYL), or the hydroxypyridinium cross-links Pyr or Dpy. 1) Much of the hydroxyproline released is in the form of free hydroxyproline, much of which is reabsorbed by the renal tubules and subsequently oxidized in the liver. Hydroxyproline-containing peptides are cleared by the kidneys and constitute the remainder of the collagen breakdown products. Originally from collagen breakdown, these small peptides are dialyzable (D) in urine. 2) In normal urine, the hydroxylysine glycosides constitute approximately 80% of the total hydroxylysine content, 10% is free, and the remainder is peptide-bound. 3) Circulating products containing the cross-links may range in size from a few amino acids to large N-telopeptide and C-telopeptide regions. Fragments of sufficiently small molecular weight are cleared by the kidneys and detected in the urine. To what degree the liver and kidney are involved in the degradation process is unclear.

Formative markers: 1) Some circulating peptides of type I collagen of bone are specific to osteoblastic activity (PICP and PINP). Once secreted, the N- and C-terminal extension peptides are cleaved by specific peptidases and these extension peptides can enter the circulation. A small amount of the N-terminal peptides, however, may bind to bone matrix. Some of the nondialyzable (ND) fraction of hydroxyproline peptides found in urine can be derived from PINP resorbed from bone matrix or cleaved from the native collagen. Both PICP and PINP are cleared by the liver. 2) The majority of osteocalcin is deposited into bone matrix while a small mount spills over into the circulation. The protein is cleared by renal catabolism, liberating free Gla and Gla-peptides into the urine. Circulating fragments of osteocalcin can be detected in blood, the origin of which is not clearly defined. 3) Serum ALP activity reflects translation of mRNA but it is not known whether changes in phospholipase activity or the physical state of the phospholipid bilayer influence levels of alkaline phosphatase activity in particular disease states.

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wheat germ lectin, heat, or chemical inactivation; Dpy by HPLC or ELISA; hydroxyproline) and a variety of species-specific antibodies to osteocalcin have been developed (dog, mouse, rat) and are commercially available (Bio Medical Technologies, Stoughton, MA).

Most investigations have focused on bone markers in human disease and have compared subject groups to seek associations between a particular marker and a physiological state, disease, response to therapy, dynamic or static histological parameters, or bone density. Such studies help to validate markers. Table 5 provides a summary of expected changes in the levels of bone markers in various disease states. More recent studies have focused on markers as tools for understanding bone physiology during intervention with known therapeutic strategies or to determine the efficacy of a particular drug in clinical trials. It is clear that simultaneous measurements of different markers, both formative and resorptive, have the potential to provide more information than would be gained from the use of only one marker. Resorptive markers may give specific information regarding the therapeutic success of antiresorptive drugs, but simultaneous measurement of formation markers will establish that the osteoblast is still active. Although all widely available markers of bone resorption measure collagen breakdown, the measurement of TRAP can also provide indirect estimation of osteoclastic activity. On the other hand, each formation marker evaluates a different osteoblastic function. With increased understanding of the metabolic fate of PICP and the precise functions of ALP and osteocalcin as well as the factors that govern their biosynthesis and metabolic clearances, abnormalities in specific osteoblast activities may be discerned and drug design can be targeted to specific functions.

B. Patient evaluation

Interest has focused on using biochemical bone markers in evaluating acute changes in bone metabolism during overt disease, during intervention with therapeutic agents, and during normal aging in individual patients. Figure 9 illustrates the complexity of all metabolic factors that contribute to the measurement of the resorptive and formative bone markers in serum and urine. Laboratory tests that have traditionally been used for the diagnosis and treatment of metabolic bone disease were largely restricted to endocrine studies of bone mineral homeostasis, particularly those related to the calciotropic hormones—vitamin D and its metabolites, PTH and calcitonin. In disorders that do not perturb hormonal status, the ability to monitor disease or judge the response to therapy has been extremely limited. Although no bone marker has been shown to be useful for diagnosis of any particular metabolic bone disease, bone markers have potential use for screening bone turnover in women at the menopause, for selection of appropriate therapy in cases of proven bone loss, and for monitoring the response to therapy.

1. Prediction of bone loss. The development of osteoporosis depends both on peak bone mass and on the rate of bone loss after the menopause, and serial measurements of bone mineral density have been used to predict future fractures. However, the interval between measurements must be of sufficient length to ensure precision. Baseline bone mineral content in combination with biochemical markers of bone turnover can be a useful predictor of future bone loss (429). Several studies cited here have correlated individual bone markers with histomorphometric or calcium kinetic measures of bone formation and/or resorption. These studies generally show a good correlation ($P < 0.05$) between these dynamic parameters and the bone marker tested *in the study population*, but the 95% confidence intervals are wide, limiting the ability to correctly classify patients into subgroups and to define turnover rate. Aside from the technical error attributable to a specific assay, intraindividual biological variations for the bone markers vary from 10–60% (Table 6). Several investigators have devised mathematical models employing combinations of markers for assessing bone turnover and predicting bone loss (429, 430). An algorithm based on serum total ALP, osteocalcin, urinary hydroxyproline, and calcium/creatinine excretion increased the correlation between estimated and measured rate of bone loss, but in general the rate of bone loss was underestimated and in some individuals this difference was great (431). Future studies using the newer markers of bone formation and resorption may improve the reliability of these models.

2. Therapeutic design and follow-up. Many different factors contribute to the acceleration in bone loss. It is likely that optimal treatment will be dependent on accurate assessment of risk factors, early diagnosis of bone loss, and an individualized therapeutic strategy. It is in this context that the bone markers are of substantial benefit. If drugs that suppress or

TABLE 5. Observed changes in bone markers in disease states

	Hyp	GHYL	Cross-links[b]	TRAP	B-ALP	Osteocalcin	PICP
Osteoporosis[a]	I	I	I	I	I	I	N
Osteomalacia	I	nk	I	nk	I	I/N	I
Primary HPT	I	I	I	I	I	I	I
Hypoparathyroidism	N	nk	D	nk	N	D	N
Paget's disease	I	I	I	I	I	I	I
Hyperthyroidism	I/N	I	I	I	I	I	N
Renal osteodystrophy	I	I	I	I	I	I	I
Bone metastases	I	I	I	I	I	I	N
Glucocorticoid excess	I/N	nk	I	nk	I	D	D

I, Increased; D, decreased; N, no change; not known.
[a] High turnover states.
[b] Includes Dpy, NTx, and CTx.

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TABLE 6. Within subject reproducibility of bone markers

Marker	% CV
NTX	20.2
Dpy (HPLC)	62.9
Hyp	53.0
Osteocalcin	27.3
ALP	10.3

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stimulate formation are indicated for a particular patient, the response to therapy may be individually monitored with such measurements. A patient with a low bone mass and a high rate of turnover will have rapid bone loss but should respond to antiresorptive agents such as estrogen/progestin, bisphosphonates, or calcitonin. On the other hand, patients with low bone turnover will have slow loss of bone and can be anticipated to have limited response to these agents. The biochemical markers of bone have proved to be very sensitive measures of acute changes in bone metabolism. Changes in the bone markers can occur within days to weeks of initiation of therapy, much sooner than can be determined from bone density measurements (usually on the order of 6 months to 1 yr). Dosage adjustments can be made rapidly based upon an individual's biochemical response.

VI. Summary and Conclusions

An ideal battery of tests would include indices of bone resorption and formation. They should be unique to bone, reflect total skeletal activity, and should correlate with traditional measures of bone remodeling activity, such as radiocalcium kinetics, histomorphometry, or changes in bone mass. Factors that confound their measurement, such as circadian rhythms, diet, age, sex, bone mass, liver function, and kidney clearance rates, should be clearly defined (Fig. 9). To date, no bone marker has been established to meet all these criteria, and each marker may have its own specific advantages and limitations. There are still questions that must be answered before there can be complete confidence in the information gained from measurement of any of the bone markers. Furthermore, it should be emphasized that none of the markers are diagnostic for any particular bone disease and cannot be used for this purpose in individual patients. Nevertheless, recent advances in research and development have provided assays with increased specificity, sensitivity, and availability. Because of this, bone markers can be used for a variety of important purposes: as tools for basic bone biology research, for defining general physiological phenomenon in clinical studies or drug trials, and for following individual patients.

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13th International Symposium of The Journal of Steroid Biochemistry & Molecular Biology

RECENT ADVANCES IN STEROID BIOCHEMISTRY & MOLECULAR BIOLOGY

May 25–28, 1997—MONACO

The 13th International Symposium of the Journal of Steroid Biochemistry & Molecular Biology—"*Recent Advances in Steroid Biochemistry & Molecular Biology*" will be held in Monaco, on May 25–28, 1997. The following topics will be considered:

1. Receptors, Structure and Gene Regulation
2. Steroids and Cancer (Including Anti-Steroids, Growth Factors, Oncogenes, and Apoptosis)
3. Steroids in the Central and Peripheral Nervous Systems
4. Enzymatic Systems and their Expression in Steroid Metabolism
5. Recent Developments of Progestins and Anti-Progestins (Including Clinical Applications)

Lectures (approximately 25–30) will be by invitation of the Scientific Organizing Committee and, in addition, there will be a poster section. All poster presentations will be subject to selection by the Scientific Organizing Committee and abstracts (maximum 200 words) must be sent to Dr J.R. Pasqualini by Monday, January 6, 1997 (postmark) at the latest (Original + 12 copies). Theramex Laboratories will award a prize of 25.000 French Francs (approx. 5,000 US$) to the best poster presentation. The total number of participants will be limited to 200. For further details, please contact:

General Scientific Secretariat: Dr J. R. Pasqualini, Steroid Hormone Research Unit, 26 Boulevard Brune, 75014 Paris, France. Tel.: (33) (1) 45 39 91 09; Fax: (33) (1) 45 42 61 21.

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