Bone Matrix Turnover and Balance In Vitro

II. THE EFFECTS OF AGEING

BARRY FLANAGAN and GEORGE NICHOLS, JR.

From the Department of Medicine, Harvard Medical School, Boston, Massachusetts 02115, Peter Bent Brigham Hospital, Boston, Massachusetts 02115, and Boston City Hospital, Boston, Massachusetts 02118

ABSTRACT The rates of both formation and resorption of bone collagen may be accurately quantitated by kinetic analysis of hydroxyproline metabolism in vitro. Using this approach we have studied the changes in bone collagen turnover with age in the rat. The rates of synthesis and resorption of collagen decline with age although the resorptive activity per cell increases up to 6 months of age. The solubility of collagen declines with age. The fraction of the newly synthesized collagen which is deposited as matrix declines dramatically with age revealing a new and hitherto unsuspected aspect of the osteoporotic process. The collagen balance becomes progressively more negative over the 1st 6 months of life. These results indicate that even in an animal who is not subject to clinical osteoporosis, biochemical measurement reveals that such a trend exists. The application of this approach to human subjects is feasible and has important implications.

INTRODUCTION

Osteoporosis is perhaps best defined as a state in which the skeletal mass is reduced below normal without alteration in its gross chemical composition. As bone collagen is turned over continuously (1), osteoporosis must always develop whenever the formation rate is exceeded by the resorption rate for a significant length of time. This will obviously hold true whatever the actual formation rate may be. The occurrence of osteoporosis in a variety of conditions such as thyrotoxicosis, Cushing's disease, osteogenesis imperfecta, immobilization, and old age make it clear that the osteoporotic state is not a single entity but a syndrome. Attempts to demonstrate a definite change in bone formation rate in one category of osteoporosis (the postmenopausal or ageing variety) in humans have indicated that this rate may be high, low, or normal (2). It seems likely therefore that even in this clinically isolated type there exists a variety of biochemical disturbances.

The absence of a reliable and accurate method for measuring the resorption rate of bone has heretofore prevented effective attack on the problem of dividing the syndrome into more logical pathophysiological categories. However, the recent development in this laboratory of a method by which the resorption and formation of bone matrix may be simultaneously and accurately quantitated in vitro (3) opened the way to a reexamination of this problem.

The present study illustrates the results obtained when this method is applied to the evaluation of changes in bone formation and resorption which accompany the ageing process in the rat.

Received for publication 15 May 1968 and in revised form 31 October 1968.

METHODS

Male Charles River rats (Sprague-Dawley strain) ranging in age from 3 to 44 wk were used in these experiments. The animals were fed a normal laboratory diet and allowed water ad lib. The rats were sacrificed by decapitation and exsanguinated.

Preparation of tissues. Metaphyseal bone from the upper tibia and lower femur was harvested by methods previously described (4). The bone was diced into pieces approximately 2 mm^3, washed vigorously three times in chilled (2°C) Krebs-Ringer bicarbonate medium buffered to pH 7.4 with 95% O_2: 5% CO_2. Bone from animals of the same age was pooled and divided into weighed aliquots before incubation. Further aliquots from each pool were set aside to determine the deoxyribonucleic acid (DNA) content of the individual pools in relation to the collagen content of the tissue.

Incubation. Tissues prepared as described were incubated using Krebs-Ringer bicarbonate medium (K-R HCO_3) buffered to pH 7.4 with 5% CO_2 and aerated with 95% O_2. Incubation volume was 2.0 ml. The medium was fortified with glucose 11.1 mM and proline 1.0 mM. Proline-U-^{14}C was added to the incubation medium at a concentration of 1.0 μc/ml.

Incubations were carried out in sealed 25-ml Erlenmeyer flasks on a Dubnoff metabolic incubator, under 95% O_2: 5% CO_2 at 37.5°C. The flasks were continuously shaken at 100 oscillations/min, for a total period of 5 hr. The samples were gassed for the 1st 15 min of incubation. The medium was changed at hourly intervals, the flasks being removed from the incubator, the medium decanted, and immediately replaced by an equal volume of fresh medium preheated to 37.5°C. Each transfer occupied less than 30 sec and regassing of the resealed flask was carried out for a further 15 min. The earlier media were stored at 2°C during the remainder of the incubation.

Analyses. After incubation the samples were removed onto ice and the final media decanted. The media from the 1st 3 hr and those from the last 2 hr were now combined to make two separate medium pools for each sample, centrifuged at 2000 rpm at 2°C for 10 min to remove any loose cells and other debris, and the supernate stored at 2°C until analyzed. The matrix samples were rinsed twice in 20 times their volume of nonradioactive K-R HCO_3 and the total washed matrix samples together with aliquots of all post-incubation media were hydrolyzed overnight in 5.7 N HCl at 115°C.

After hydrolysis the samples were cleaned with a resin-charcoal mixture and analysis of aliquots of the cleaned hydrolysates for total hydroxyproline content was carried out by the method of Prockop and Udenfriend (5). The determination of DNA was carried out on 0.1 N NaOH extracts of the bone chips as previously described (6) by the hot trichloracetic acid extraction procedure of Schneider (7).

Isolation of hydroxyproline radioactivity from aliquots of the hydrolysates was carried out using the silicic acid column of Juva and Prockop (8). Medium proline was determined using the method of Chinard (9).

Radioactive counting. All counting was done in an automatic liquid scintillation spectrometer (Packard-Tricarb 314E; machine efficiency for ^{14}C ranging from 70 to 72%) as previously described (3). Internal standardization was carried out individually for all aqueous samples. Recoveries ranged from 80 to 95%.

Calculations. We have described in detail elsewhere (3) the features of the accumulation of hydroxyproline in incubation medium and matrix, which allow the simultaneous quantitative determination in this system of the rates of (*a*) collagen synthesis, (*b*) deposition of matrix collagen, (*c*) resorption of matrix collagen, and (*d*) the passive solubility of matrix collagen.

RESULTS

A. Solubility. In Fig. 1 is outlined the effect of ageing on the relative solubility of bone collagen. It is clear that the solubility of bone collagen is extremely low at all ages. Nevertheless, there is an acute decrease in solubility between the ages of 6 and 13 wk, and this becomes progressively more marked with further ageing.

B. Balance. The balance of collagen synthesis vs. collagen resorption over the age range studied is plotted in Fig. 2. The synthesis and resorption rates are expressed in terms of the collagen content of the samples. The figure illustrates that in this in vitro system the *total collagen* synthesized exceeds the total collagen resorbed up to the age of 6 wk, resulting in a positive balance during this active growth phase. By 13 wk, the balance between these two quantities has become negative, and it remains so up to 44 wk. The shift from positive to negative balance is caused by a progressive reduction in the synthetic rate and occurs despite a progressive reduction in the resorption rate, which diminishes in similar fashion but to a lesser degree with ageing.

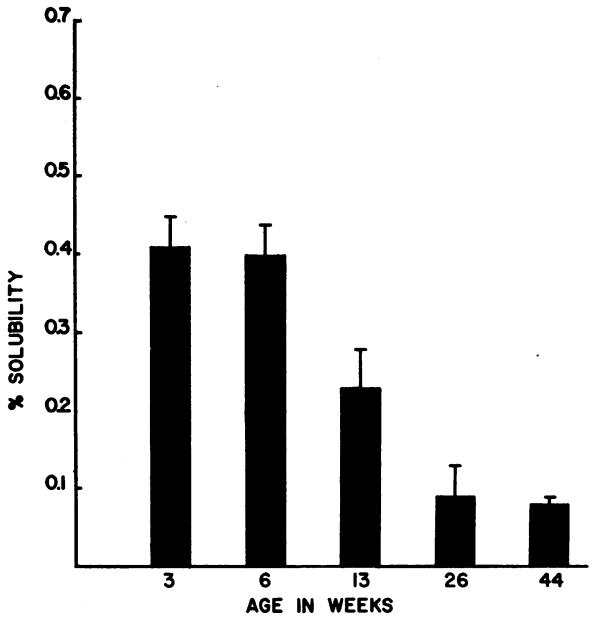

FIGURE 1 Changes in the passive solubility of calcified bone collagen with ageing. One SD is shown. The solubility is less than 0.5% at 3 wk and falls to about half this value ($P < 0.001$) by 13 wk. There is a progressive fall with ageing. The 44 wk value is reduced to 56% of the 13 wk value ($P < 0.001$).

However, as all of the collagen which is synthesized during the in vitro incubation is not in fact deposited (3), a more accurate reflection of tissue collagen balance is obtained by plotting the total new collagen *deposited in matrix* against the collagen resorbed. These data are shown in Fig. 3 and differ from those outlined in Fig. 2 in that a minor degree of negative balance is present throughout.

C. Deposition ratio. The differences between Figs. 2 and 3 are due to the incomplete deposition of the newly synthesized collagen. What is not shown clearly by the plots, however, is the remarkable change in the degree of deposition of newly synthesized collagen which occurs with ageing. In Fig. 4 the deposition ratio (i.e. the ma-

trix collagen deposition rate, expressed as a percentage of the total collagen synthetic rate) at different ages is shown. The level is maintained at a relatively stable value of 60–65% up to 3 months of age and then declines precipitously thereafter to a 10 month mean value of 13.8%. In other words, not only is the total collagen synthesized grossly reduced with ageing, but in addition, the proportion of the newly synthesized collagen which is deposited falls by 44 wk to about 20% of the rate shown in the younger age groups.

D. Cellular rates vs. tissue rates. We turn finally to the contrast between the rates of matrix formation and resorption when they are expressed on a cellular basis on the one hand, and on a tissue collagen basis on the other.

The matrix formation rate plotted against these differing bases is shown in Fig. 5. Clearly, not only is the tissue renewal rate (Fig. 5 B) progressively reduced with age, but so also is the amount of collagen synthesized per mg of DNA (the cellular formation rate). However, when we consider resorption activity in a similar fashion a different pattern emerges (Fig. 6). The cellular resorption rate (Fig. 5 A) increases progressively from 6 wk to 26 wk and then returns to the initial level by 44 wk, whereas the tissue resorption rate falls continuously with advancing age.

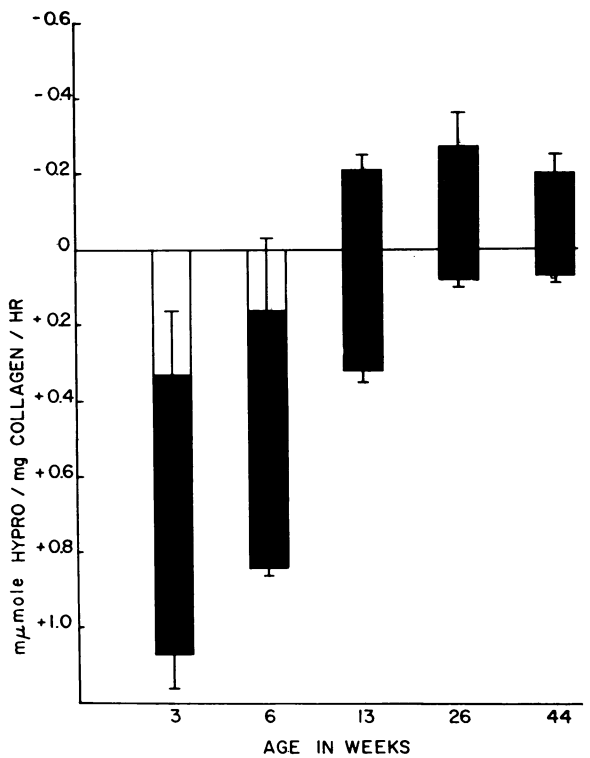

FIGURE 2 The collagen balance of living bone in vitro. The collagen synthetic rate is compared with the collagen resorption rate, each being referred to the collagen mass of the tissue. The mean synthetic rate is plotted downwards from the balance (zero) line; the mean resorption rate is then plotted upward from the mean synthetic rate value. The balance is positive or negative by the amount whereby the resorption rate fails or succeeds in crossing the balance line. One SD is shown, downward for synthesis and upward for resorption. Hypro = hydroxyproline.

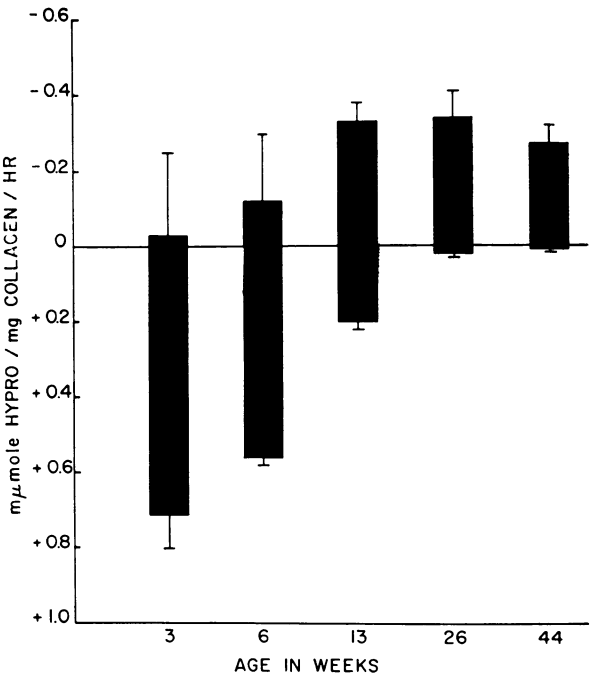

FIGURE 3 The collagen balance of living bone in vitro. The deposition rate of newly formed collagen in matrix is compared with the collagen resorption rate, each being referred to the collagen mass of the tissue. The mean deposition rate is plotted downward from the balance line; the mean resorption rate is then plotted upward from the mean deposition rate value. The balance is negative or positive according as the resorption rate fails or succeeds in crossing the balance line. One SD is shown, downward for synthesis and upward for resorption. Hypro = hydroxyproline.

DISCUSSION

The expectation that ageing will result in a variety of demonstrable changes in bone collagen metabolism is borne out by these results. The changes affect not only the more generally recognized features of metabolism such as formation and resorption rate, but also the physicochemical solubility of collagen, and quite unexpectedly, the proportion of newly synthesized collagen which is deposited.

The over-all solubility of calcified bone tissue is is known to be very low (10) and indeed the calcified matrix is not attacked to any appreciable extent by a variety of proteolytic enzymes, including collagenase (11). In these experiments considerably less than 1%

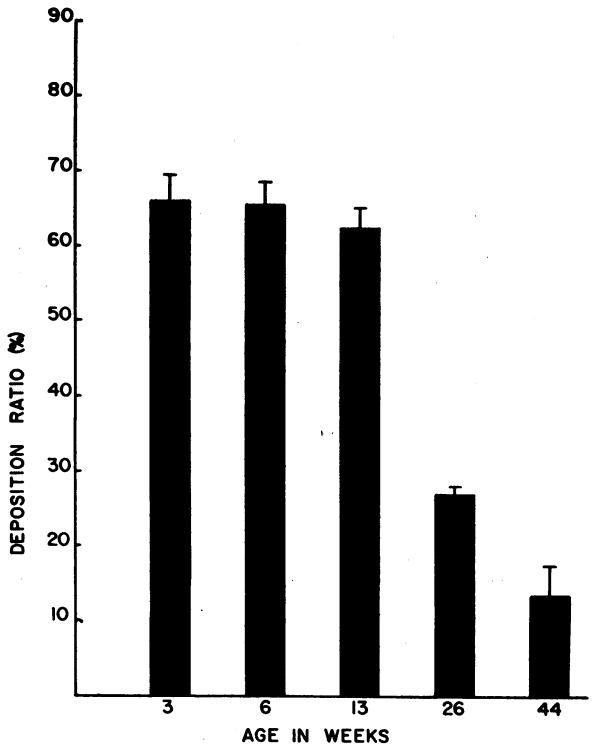

FIGURE 4 The changes in bone collagen deposition ratio with ageing. The fraction of the total newly synthesized collagen which is actually deposited in matrix is expressed as a percentage of the total collagen synthesized. One SD is shown. There is no significant difference between 3 wk and 13 wk of age. By 26 wk a precipitous fall to less than 50% of the mean 13 wk level has occurred ($P < 0.001$ and by 44 wk a further fall to 50% of the 26 wk level ($P < 0.001$) occurs.

of the total collagen pool is seen to go into solution under physiologic conditions, and this small fraction progressively decreases with age. A substantial body of evidence indicates that the more recently synthesized forms of collagen are more soluble (12). Alterations in solubility which we have observed in other conditions of diminished synthesis make it likely that the depression in collagen solubility which occurs with ageing is to some extent a reflection of the diminished synthetic rate although the falling resorption rate may also contribute to this reduction in the soluble pool. It should be borne in mind that we are discussing the solubility of the total collagen present at zero time, and that this in fact is a solubility pool which will reflect prior synthetic and resorptive activity indirectly.

The fall in the deposition ratio with advancing age has hitherto been unsuspected, and implies that the efficiency of the deposition process which leads to matrix formation is adversely affected by the ageing process. The extent of this decline is a major one. In fact, if the deposition ratio were not to fall with ageing, the matrix formation rate at 44 wk would be 500% of the rate actually measured. The cause of this change is unclear and obviously relates to the organization of newly synthesized collagen in the extracellular area. It may be associated with other changes in synthesis by the cell, perhaps in the mucoprotein components, which presumably play a part in the organization of the matrix. Whatever the cause, it does appear to be a significant phenomenon in the normal evolution of the skeleton with age.

The existence of a positive balance of synthesis over resorption during the 1st 6 wk is the pattern to be expected in bone taken from very young animals and the change to a negative balance at around 13 wk coincides with the achievement of chemical maturity (13). However, the actual matrix balance, as determined by the comparison of deposition vs. resorption (Fig. 3) is negative in all groups studied.

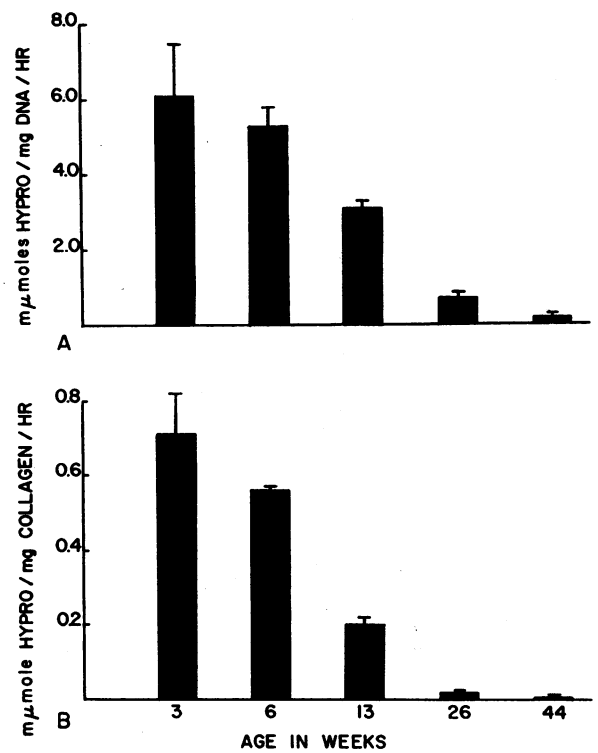

FIGURE 5 The changing rate of formation of bone matrix collagen with age expressed on two different bases. In A the formation rate is expressed against the DNA content of the tissue and in B against the tissue collagen content. One SD is shown. The pattern is similar in both instances. A progressive and continuously significant fall in matrix formation rate occurs throughout this age range. No significant difference in pattern was noted when total collagen synthesis was plotted instead of matrix formation rate. A reflects the average synthetic activity per cell, B gives a true measure of the tissue collagen replacement rate.

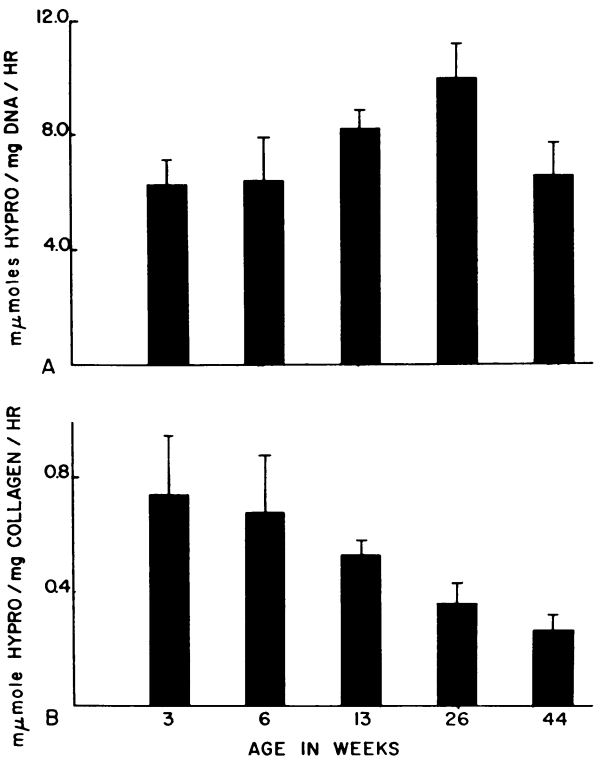

FIGURE 6 The changing rate of resorption of bone collagen with age expressed on two different bases. In A the rate is expressed against the DNA content of the tissue, and in B against the tissue collagen content. One SD is shown. The patterns are dissimilar, the cellular resorption rate rising moderately but significantly at 13 and 26 wk and falling again to a baseline value at 44 wk.

By contrast the tissue resorption rate decreases progressively throughout this age range. A reflects the average resorptive activity per cell, whereas B is a true reflection of the actual tissue resorption rate.

This is not surprising, when we consider that this preparation represents in fact the immediate postfracture metabolic status of the tissue. It has been pointed out by Bauer (14) that the immediate result of fracture is an increase in bone resorption rate which is subsequently followed by a stimulus in formation. We have ourselves observed in a similar system to the one used in this study, that the immediate effect of subcapital fracture on the synthetic rate of collagen in the dog femur is a reduction in this rate on *both* sides of the fracture line (15). Consequently, one may expect a shift in the balance towards the negative side in studies of this type. It is clear that such a shift is not of gross degree, although it does result in a baseline shift from the in vivo situation. Furthermore, it is worthy of mentioning here that additional studies using this system have shown that a clear-cut shift in collagen balance from negative to positive is achieved in vitro using moderately elevated medium phosphate concentrations.[1] It is therefore possible by varying in vitro conditions, to set the balance point at any level which seems appropriate.

The necessity to distinguish between standards of reference used in the interpretation of these results is emphasized by the comparison of resorption rates and formation rates outlined in Figs. 5 and 6. The relationship of DNA (cell number), collagen content (substrate amount), and wet weight of bone is quite constant in any given age group. However, variations in the relationship of DNA to the other parameters occurs in many circumstances, including ageing, where a moderate but definite decrease in cellularity is noted (16). In such circumstances the apparent rates of dynamic processes may well vary depending on the entity to which they are referred. This has been pointed out previously in a study of human bone samples where the cellularity may vary over a very large range (17).

The crucial distinction, however, that is to be made is that the meaning of the data is quite different depending upon the standard of reference. When the cellularity (DNA) is used, we are contrasting the mean formation or resorption activity per tissue cell, thereby getting an average figure for the rate of cellular formative or resorptive activity; this does not necessarily reflect the over-all tissue economy which is seen only when the rate processes are referred to the initial substrate mass of collagen which is being turned over. This latter is the important value in determining the presence of positive or negative balance and consequently of the tendency toward osteoporosis. These considerations are underlined by Figs. 5 and 6. Whereas the formation rate follows the same trend both per cell and per unit collagen, the resorptive activity per cell increases up to 6 months, while resorption rate of the tissue decreases progressively during this time.

Although the rat is not noted for the development of symptomatic osteoporosis with advancing age, it is clear that a tendency towards negative balance with ageing occurs in this animal. This is associated with a progressive reduction in matrix formation which is due to a depression of collagen synthesis and is exacerbated by a failure to deposit in matrix the same proportion of newly formed collagen as occurs at earlier ages. The resorption rate is also reduced progressively but to a lesser degree.

In this description we have a precise formulation of the evolution of an osteoporotic state in terms of these rate processes. In a similar fashion the actions of other agents known to produce osteoporosis may be evaluated, and the areas of major derangement isolated for further study. Clarification of whether these derangements are at a

[1] Flanagan, B., and G. Nichols, Jr. The effect of phosphate on bone matrix balance in vitro. Manuscript in preparation.

subcellular level involving the mechanics of a single rate process or at a supracellular level involving the modulation of osteoblastic and osteoclastic activities in an orchestrated fashion remains to be determined. The latter hypothesis which has been advanced by Frost (18) as a result of this group's painstaking quantitative histologic studies is at once the most likely explanation and the most difficult to evaluate directly.

The future study of metabolic bone disorders in man requires the ability to evaluate quantitatively as well as qualitatively the living biopsy sample. The present technique has been developed to meet such a need. That it can do so is illustrated by the results obtained in this study, as well as by previous work on human biopsy samples (19). Data acquired in this fashion complement the other major methods in current use including mineral kinetic studies, mineral balance studies, and the various forms of radiologic and histologic assessment. It supplies over and above these methods the ability to quantitate repetitively and directly matrix formation, deposition, and resorption rates.

ACKNOWLEDGMENTS

The authors wish to express their gratitude for the skillful technical assistance of Mrs. Susan Ault, Miss Mimi Bowler, and Miss Peggy Rogers.

This work was supported in part by the U. S. Public Health Service Grants AM 00854-12 and -13, and in part by a grant from the John A. Hartford Foundation.

REFERENCES

1. Gerber, G., G. Gerber, and K. I. Altman. 1960. Studies of the metabolism of tissue proteins. I. Turnover of collagen labelled with proline-U-^{14}C in young rats. *J. Biol. Chem.* **235**: 2653.
2. Nichols, G., Jr., and B. Flanagan. 1966. Osteoporosis—A disorder of bone cell metabolism. *Fed. Proc.* **25**: 922.
3. Flanagan, B., and G. Nichols, Jr. 1968. Bone matrix turnover and balance in vitro. I. The effects of parathyroid hormone and thyrocalcitonin. *J. Clin. Invest.* **48**: 595.
4. Borle, A. B., N. Nichols, and G. Nichols, Jr. 1960. Metabolic studies of bone in vitro. I. Normal bone. *J. Biol. Chem.* **235**: 1206.
5. Prockop, D. J., and S. Udenfriend. 1960. A specific method for the analysis of hydroxyproline in tissues and urine. *Anal. Biochem.* **1**: 228.
6. Flanagan, B., and G. Nichols, Jr. 1962. Metabolic studies of bone *in vitro*. IV. Collagen biosynthesis by surviving bone fragments *in vitro*. *J. Biol. Chem.* **237**: 3686.
7. Schneider, W. C. 1957. Determination of nucleic acids in tissues by pentose analysis. *In* Methods in Enzymology. S. P. Colowick and N. O. Kaplan, editors. Academic Press Inc., New York. **3**: 680.
8. Juva, K., and D. J. Prockop. 1966. Modified procedure for the assay of ^{3}H- and ^{14}C-labelled hydroxyproline. *Anal. Biochem.* **15**: 77.
9. Chinard, F. P. 1952. Photometric estimation of proline and ornithine. *J. Biol. Chem.* **199**: 91.
10. Neuman, W. F., B. J. Mulryan, and G. R. Martin. 1960. A chemical view of osteoclasts based on studies with ytrium. *Clin. Orthop. Related Res.* **17**: 124.
11. Howes, E. L., I. Mandl, S. Zaffuto, and W. Ackermann. 1959. The use of clostridium histolyticum enzymes in the treatment of experimental third degree burns. *Surg. Gynecol. Obstet.* **109**: 177.
12. Jackson, D. S. 1957. The Formation and Breakdown of Connective Tissue. *In* Connective Tissue. R. E. Tunbridge, editor. Charles C Thomas, Publisher, Springfield, Ill. 62.
13. Spray, C. M., and E. M. Widdowson. 1950. The effect of growth and development on the composition of mammals. *Brit. J. Nutr.* **4**: 332.
14. Bauer, G. 1964. Kinetics of bone disease. *In* Bone Biodynamics. H. M. Frost, editor. Little, Brown & Co., Inc., Boston. 489.
15. Cronk, R., B. Flanagan, G. Nichols, Jr., and H. Banks. 1968. Metabolic and histologic changes in femoral neck fractures leading to aseptic necrosis. *Surg. Forum.* In press.
16. Nichols, G., Jr. 1962. Differential "ageing" in two types of bone. *J. Clin. Invest.* **41**: 1389. (Abstr.)
17. Flanagan, B., and G. Nichols, Jr. 1965. Metabolic studies of human bone *in vitro*. I. Normal bone. *J. Clin. Invest.* **44**: 1788.
18. Frost, H. 1964. The dynamics of bone remodelling. *In* Bone Biodynamics. H. M. Frost, editor. Little, Brown & Co., Inc., Boston. 315.
19. Flanagan, B., and G. Nichols, Jr. 1965. Metabolic studies of human bone in vitro. II. Changes in hyperparathyroidism. *J. Clin. Invest.* **44**: 1795.