Bone Matrix Turnover and Balance In Vitro

I. THE EFFECTS OF PARATHYROID HORMONE AND THYROCALCITONIN

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ABSTRACT Labeled proline from incubation media has been shown to be incorporated into living bone matrix collagen in vitro. Hydroxyproline is released from fresh bone slices in similar systems in a characteristic curve against time. This hydroxyproline is derived from three distinct sources, each of which may be separately quantitated. Part of the total represents passive solubilization of matrix collagen, part is derived from new synthesis of soluble collagen occurring in vitro, and the remainder is released by cell-mediated resorptive action.

The latter two processes are linear with time up to 8 hr; the former decays to zero at about 2 hr. Consequently, rates of collagen synthesis and of new collagen deposition and resorption can be quantitated simultaneously in the same system. The ability to measure these parameters of bone collagen metabolism provides methods both for the accurate evaluation of organic matrix resorption in vitro and for the accurate measurement of rates of collagen synthesis and collagen deposition. The application of the method is illustrated using parathyroid hormone and thyrocalcitonin. Parathyroid hormone diminishes collagen synthesis and stimulates collagen resorption. It reduces slightly the deposition of newly formed collagen in stable matrix. The net effect of these changes is to produce a marked negative balance. It does not significantly affect the solubility of matrix collagen.

Thyrocalcitonin does not affect collagen synthesis or its deposition. It causes a marked fall in resorption rate. It has no effect on matrix collagen solubility. The net effect is to produce a marked positive balance of matrix collagen.

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INTRODUCTION

The examination of various aspects of bone metabolism may be carried out with advantage at the tissue slice level (1–5). Bone turns over at a rate which is determined by two separate processes, those of resorption and accretion. The cellular fraction of bone, although unimportant in mass, is believed to control the rates at which these two processes take place, and the existence of a relatively stable skeletal mass throughout a long period of adult life suggests that the rates of these two processes are, at least crudely, coupled.

Considerable information concerning both the rate of accretion of bone and details of synthesis of matrix components (6–8) has been accumulated, although much still remains to be clarified concerning the ultrastructural organization of matrix and the process of calcification. By contrast, understanding of the processes involved in bone resorption has lagged far behind. This has been due in no small measure to the absence of a reliable, rapid, and direct method of quantitating the resorption rate of bone. The ability to detect changes in resorption rate is a prerequisite to further analysis of the mechanisms involved.

From a biological viewpoint resorption consists of the successive or simultaneous removal of all of the components of bone, namely organic matrix, bone mineral, and bone cells. It is unclear whether bone cells ever in fact do resorb themselves or whether they merely mi-

grate through areas of active resorption, departing when resorption is completed. Clearly the measurement of changes in bone cell mass would not reveal quantitative information concerning bone resorption rate. The mineral component, while resorbed in measurable fashion in vitro, suffers from the disadvantage that a physicochemical solubility equilibrium exists between medium and tissue, making the interpretation of measured mineral resorption rate suspect (9).

The resorption of organic matrix on the other hand is subject to no such strictures. The major component of bone's organic matrix is collagen. Hydroxyproline serves as a legitimate built-in biological label for collagen for three reasons: (*a*) It is present in significant amounts only in collagen; (*b*) it is not utilized in the synthesis of collagen; and (*c*) it makes its biological appearance only after formation of the polypeptide chain of protocollagen and under the influence of a specific cellular enzyme system (10). To these may be added the fact that in this system hydroxyproline is neither utilized nor degraded during incubation, but is recovered unchanged,[1] allowing accurate estimation of the hydroxyproline released during incubation.

The experiments presented here were designed to separate the total hydroxyproline released into the medium and synthesized in the bone sample into its component parts. Analysis of these processes permits the simultaneous direct measurement of rates of collagen synthesis, deposition, and resorption in a single bone sample in vitro. Rates of bone matrix turnover and balance can therefore be estimated using these methods. The effects of parathyroid hormone and thyrocalcitonin on these various parameters of bone collagen metabolism are demonstrated.

METHODS

Preparation of tissues. Male Charles River rats (originally Sprague-Dawley strain) ranging in age from 40 to 50 days were used throughout these experiments. The animals were killed by decapitation and exsanguinated.

Metaphyseal bone from the upper tibia and lower femur was harvested by methods previously described (1). 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, and divided into weighed aliquots before incubation.

In those experiments designed to study the relationship of resorption to cell mass and tissue mass, cortical and trabecular bone from the metaphyseal areas were separated by sharp dissection before washing and dicing, and aliquots of these pools prepared separately for incubation.

In other experiments designed to examine the specific activity of the intracellular proline pools, bone cells were isolated from bone slices after incubation by a method combining grinding, flotation, and sedimentation described in detail elsewhere (11).

[1] Listgarten, M., B. Flanagan, and G. Nichols, Jr. Unpublished observations.

Similar separation of bone cells from bone matrix was carried out in attempts to evaluate the solubility of calcified bone collagen. After separation of the bulk of the cell pool by this initial grinding step, the remaining matrix was exhaustively extracted for 5 days in the cold (2°C) with isotonic saline metastable with regard to calcium and phosphate. This material contained no measurable deoxyribonucleic acid (DNA) after completion of these extractions, thus providing an acellular calcified matrix preparation.

Incubation. Tissue prepared as described was incubated in Krebs-Ringer bicarbonate medium, buffered to pH 7.4 with 5% CO_2 and aerated with 95% O_2. Incubation volumes varied from 2.0 to 5.0 ml, representing approximately 1.0 ml of medium per 100 mg wet weight of tissue.

The medium was fortified with glucose 11.1 mM and proline, usually 1.5 mM. In those experiments involving determination of the intracellular proline specific activity and measurements of labeled hydroxyproline, proline-U-^{14}C was added to the medium at a concentration of 2.0 μc/ml.

Incubations under a 95% O_2: 5% CO_2 atmosphere were carried out in a Dubnoff metabolic incubator at 37.5°C with continuous shaking at 100 oscillations/min for periods up to 8 hr. Samples were gassed for the 1st 15 min of incubation. In experiments involving medium changes at various time intervals the flasks were removed from the incubator, the medium decanted, and immediately replaced with 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.

Hormone studies. 21-day old male rats were thyroparathyroidectomized surgically. In studying the effect of parathyroid hormone, parathormone (Eli Lilly & Co.) 1.0 unit/g body weight was administered subcutaneously 24 hr later. These animals together with their controls were sacrificed 18 hr after hormone injection. Carotido-jugular blood was collected for serum calcium determination, and metaphyseal bone harvested and incubated in the usual fashion. The effect of thyrocalcitonin was studied by the application of the hormone in vitro, as this was found to give the most clear-cut response, for reasons which will be discussed elsewhere.[2] After preparation of bone as usual from 21-day old animals surgically thyroparathyroidectomized 24 hr before sacrifice, the hormone was added to the medium in a dose of 10 M.R.C. mU/ml. This hormone level was renewed each hour, accompanying the changing of the medium. The preparation used was trichloracetic acid–precipitated porcine thyrocalcitonin,[3] assayed at 350 M.R.C. mU/mg dry weight.

Analysis. After incubation, the samples were removed onto ice; the medium was decanted and centrifuged to remove any loose cells and other debris, and the supernate stored at 2°C until analyzed. Total medium hydroxyproline was measured on aliquots which were hydrolyzed either overnight or for a minimum of 3 hr in 5.7 N HCl at 115°C (the shorter time period having been proved adequate for total hydrolysis by a separate investigation). All matrix samples were hydrolyzed overnight.

After hydrolysis the samples were cleaned with the resin-charcoal mixture recommended by Prockop and Udenfriend (12). Analysis of the cleaned hydrolysates for hydroxyproline was carried out initially using the Martin and Axelrod modification (13) of the Neuman and Logan method (14). A comparison of the results provided by this simple method

[2] Flanagan, B., and G. Nichols, Jr. Manuscript to be published.

[3] Prepared by the Armour Pharmaceutical Co. and kindly supplied by Dr. Arman Tashjian, Harvard Dental School.

with those given by the more complex method of Prockop and Udenfriend (12) *usually* revealed no significant differences. Presumably neither medium nor matrix contained sufficient concentration of interfering substances to render the Neuman and Logan method unreliable in this situation. However, when the medium hydroxyproline content fell below a certain critical amount, as for example, when the release rate was separately determined during each hour of incubation, the Neuman and Logan method was not reliable and in this situation the method of Prockop and Udenfriend (12) was used. The determination of DNA was carried out on 0.1 N NaOH extracts of the bone chips as previously described (15), by the hot tricholoracetic acid (TCA) extraction procedure of Schneider (16), and measurement of the incorporation of labeled hydroxyproline into alkali-stable collagen referred to in Fig. 8 was carried out on the cleaned collagen fraction which remains after such alkali extraction (15).

Sephadex fractionation of medium. To evaluate the relative sizes of hydroxyproline-containing peptides present in the medium at the end of incubation, aliquots of unhydrolyzed final medium were fractionated by passage through Sephadex G50 columns (90.0 × 1.5 cm). Elution was carried out with isotonic NaCl buffered with sodium phosphate to pH 7.4. The eluate was collected in 2.0 ml fractions, the end point being determined by the elution of the free proline radioactivity. The samples were then hydrolyzed and analyzed for hydroxyproline as already described.

Isolated cell proline pools. In experiments designed to study the intracellular proline pool directly the washing medium was kept at 37.5°C, as previous work has shown that proline is concentrated intracellularly by an active transport process and that such concentration gradients may be abolished by lowering the ambient temperatrue (17). The washed cells were precipitated with a final concentration of 10% TCA and spun at 2000 rpm for 10 min. The supernatant solution was analyzed for its proline content by the method of Chinard (18), and aliquots set aside for determination of proline radioactivity.

Isolation of proline and hydroxyproline radioactivity. Proline radioactivity was determined by the method of Peterkofsky and Prockop (19). This method, which in most situations permits separate determination of hydroxyproline radioactivity, was not sufficiently sensitive to detect small amounts of labeled hydroxyproline in the medium in the presence of relatively large amounts of labeled proline. Therefore, hydroxyproline label in the medium samples in earlier experiments was separated from proline by a double passage through the modified Moore and Stein column of Leach (20) followed by one-dimensional paper chromatography using phenol H_2O–NH_3 as the solvent phase. In later studies the silicic acid column method of Juva and Prockop (21) was used instead, since it proved sufficiently sensitive and considerably less time-consuming.

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%). Aqueous samples were added to 15.0 ml of scintillation fluid consisting of toluene with 4.0 g 2,5-diphenyl oxazole and 50 g *p*-bis(2,5-phenyloazolyl)benzene/liter, as primary and secondary fluors. The aqueous samples were solubilized with the aid of 1.5 ml of 1.0 M Hyamine hydroxide in methanol. Internal standardization was carried out individually for all aqueous samples. Recoveries range from 82 to 95%.

RESULTS

Preliminary attention was given to the selection of a standard of reference against which to measure tissue hydroxyproline release. Metaphyseal bone was separated into cortical and trabecular portions, and the release of hydroxyproline from these separated bone types was evaluated both on the basis of tissue collagen as calculated from tissue hydroxyproline content (13%) and of tissue cell number estimated as DNA content. The expectation that cortical and trabecular bone would have

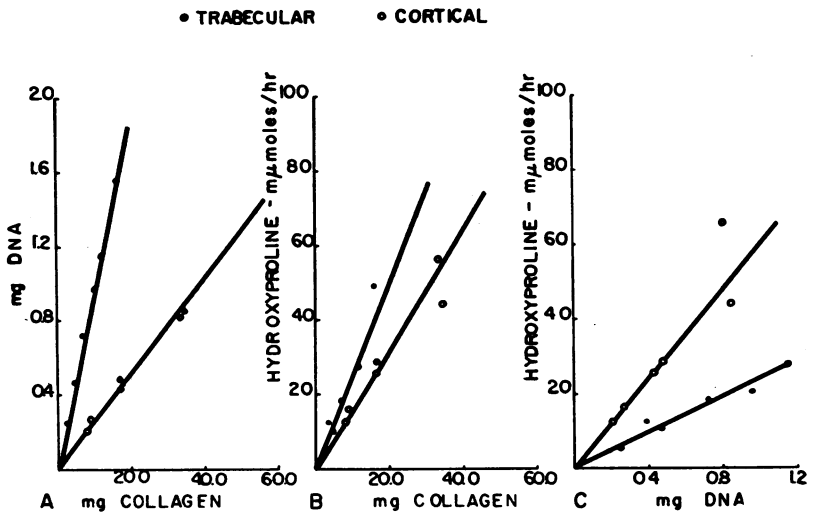

FIGURE 1 A. The relationship of DNA to collagen in cortical and trabecular bone. B. The relationship of total hydroxyproline release into medium by the same samples, plotted against tissue collagen. C. The relationship of total hydroxyproline release into medium by cortical and trabecular bone slices plotted against DNA content. Incubation time in B and C: 4 hr.

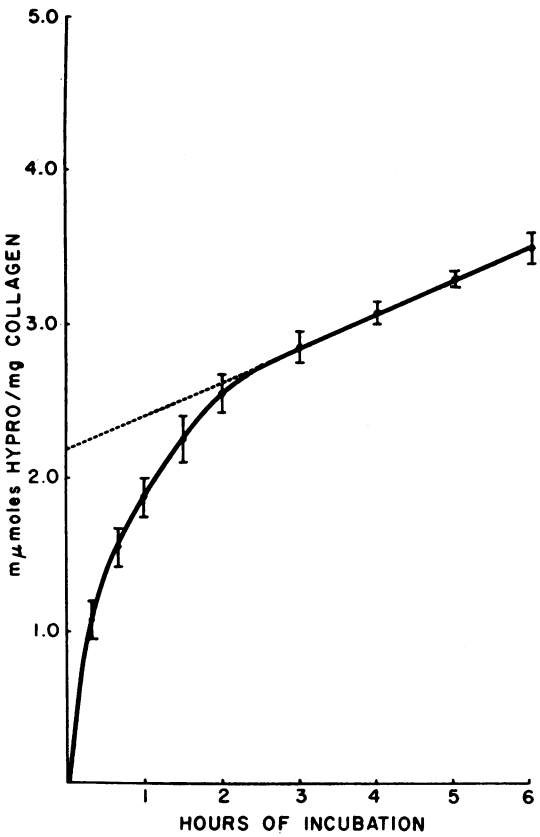

FIGURE 2 The mean cumulative release of hydroxyproline (hypro) from living bone plotted against time. Mathematical analysis of these data revealed the presence of two components, one linear and constant throughout the incubation period, and one experimental, which disappeared at about 2.5 hr. Two SEM above and below the line are shown.

quite dissimilar ratios of DNA to collagen was confirmed by the data illustrated in Fig. 1 A. The mean DNA content of trabecular bone was 96 μg/mg collagen and that of cortical bone 26 μg/mg collagen.

When the hydroxyproline releases of individual samples are plotted against both their DNA content and their collagen content (Fig. 1), an obvious difference emerges. The release of hydroxyproline in all instances is linear, whatever the reference standard chosen. There was a disparity between the bone types in the release rates both on a DNA basis and on a collagen basis. However, as the relationships to both DNA and collagen content was linear in both situations, either could be used as a reference standard when employing a reproducible system of mixed cortical and trabecular bone as in these experiments. In the remaining experiments in this series, collagen content has been employed as a reference standard.

Attention turned next to the quantification of hydroxyproline release with time of incubation. The results are illustrated in Fig. 2 in which the release rate has been plotted directly against time. A curvilinear followed by a rectilinear pattern of release was clearly observed. Analysis of this curve revealed that it could be broken down into an initial faster release which decayed exponentially to zero at around 2 hr of incubation, and a slower rate, rectilinear with time, which persisted for at least 6 hr.

The existence of this biphasic pattern suggested that medium hydroxyproline was derived from two pools and raised the question of the significance of each. Possible differences in the molecular species of hydroxyproline-containing peptides released during the earlier and later phases of incubation were, therefore, sought. However, Sephadex 50 fractionation of separately collected 0–2 hr and 2–6 hr medium hydroxyproline revealed no differences. Hydroxyproline-containing peptides of molecular weights of both larger and smaller than 50,000 were present, and approximately 50% of the total hydroxyproline was in the free state.

A second set of experiments was designed to determine whether either or both of these processes were dependent on the existence of cellular activity during the course of incubation. The release of hydroxyproline into the medium with time, from calcified but cell-free matrix prepared as described above is illustrated in Fig. 3. It is apparent that the linear rate process disappears when the cells are removed, while the exponential early release remains unaffected. Therefore, the early rapid release of hydroxyproline was taken to represent a solubility phenomenon, whereas the latter was dependent on the presence of cellular activity and might properly be considered to contain the hydroxyproline released in cell-mediated resorption. Moreover, the relative contributions of both of these processes to the total pool could be measured by simple extrapolation of the linear rate back to zero time (Fig. 2), the point of intersection with the ordinate representing the size of the soluble collagen pool.

Another fundamental problem in the interpretation of hydroxyproline release into the medium is the question of whether newly synthesized collagen hydroxyproline contributes significantly to the medium pool which accumulates during the course of incubation. Preliminary experiments using proline-U-^{14}C as a marker revealed that newly synthesized hydroxyproline could account for approximately 8% of the *total* hydroxyproline released into the medium, during 6 hr of incubation, by bone slices from rats in this age range (22). This figure was calculated on the assumption that no significant tissue pool of either free proline or collagen precursors is interposed between the labeled medium proline supplied, and the labeled medium hydroxyproline produced. Any

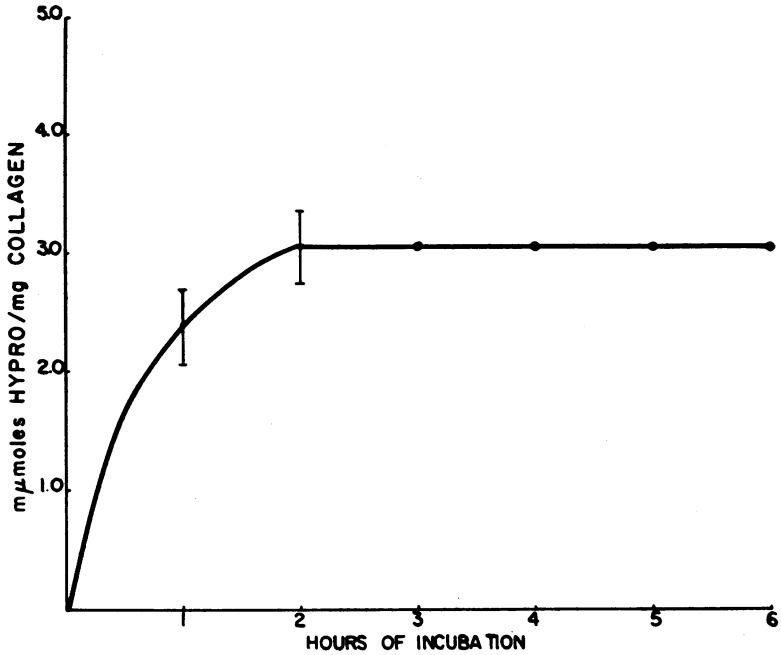

FIGURE 3 The cumulative release of hydroxyproline with time from undecalcified bone matrix freed of its cell content by grinding and saline extractions for 5 days (see Methods). DNA content was unmeasurable. The mean and one SEM above and below the line are shown. No measurable release was noted after 2 hr.

error inherent in this assumption would result in an *underestimate* of the total newly synthesized hydroxyproline of the medium, and clearly this aspect required additional clarification if the rate of cellularly mediated resorption was to be quantitated.

Potential sources of medium hydroxyproline are diagrammatically represented in Fig. 4. The total medium hydroxyproline released with time is determined by the sum of newly synthesized collagen solubilized (or perhaps not yet deposited) (K_6), the old collagen spontaneously solubilized ("K_7") which is actually a pool size, rather than a rate process, and the old collagen resorbed by cellular activity (K_8). We have already shown that "K_7" and the sum of K_6 and K_8 can be separately quantitated. Since the sum of K_6 and K_8 proved linear with time, it was likely that both processes were separately linear with time.

The exact contribution of newly synthesized collagen hydroxyproline to the medium was determined in two ways. First, we established conditions whereby the intracellular free proline pool specific activity could be made equivalent to that of the external medium, within a reasonable time period of incubation.

The cell-free proline pool was isolated after preliminary loading of the cells with exogenous proline and its specific activity measured directly as follows: bone chips were incubated in medium containing 1.5 mM proline labeled with proline-U-^{14}C. After incubation for periods ranging from 1 to 6 hr, the chips were removed, washed free of hot medium, and the cell pools individually isolated by grinding. All operations were performed at 37.5°C,, as we had previously shown that these cells concentrate free proline (11), and that the concentration gradient is temperature dependent (17). After isolation of the cell pools and precipitation with a final concentration of 10% TCA, the supernatant free proline specific activity was measured. Since the results showed no significant difference between 1 and 6 hr, all values were grouped together for statistical analysis. Mean medium proline SA measured 44,316 ±2237 (1 SD) cpm/mμmole; mean cell proline SA measured 44,114 ±7816 (1 SD) cpm/mμmole. The intracellular and medium proline specific activities were therefore found to be equal no later than 1 hr after the start of the incubation of the bone slices.

Experiments were then conducted to evaluate the possibility that the intervening pool of intermediates was sufficiently small to allow complete labeling of the collagen-forming system within the viability time of the bone slices. Incubations were carried out using 1.5 mM proline with proline-U-^{14}C added, and the labeling of hydroxyproline in the medium measured by changing the

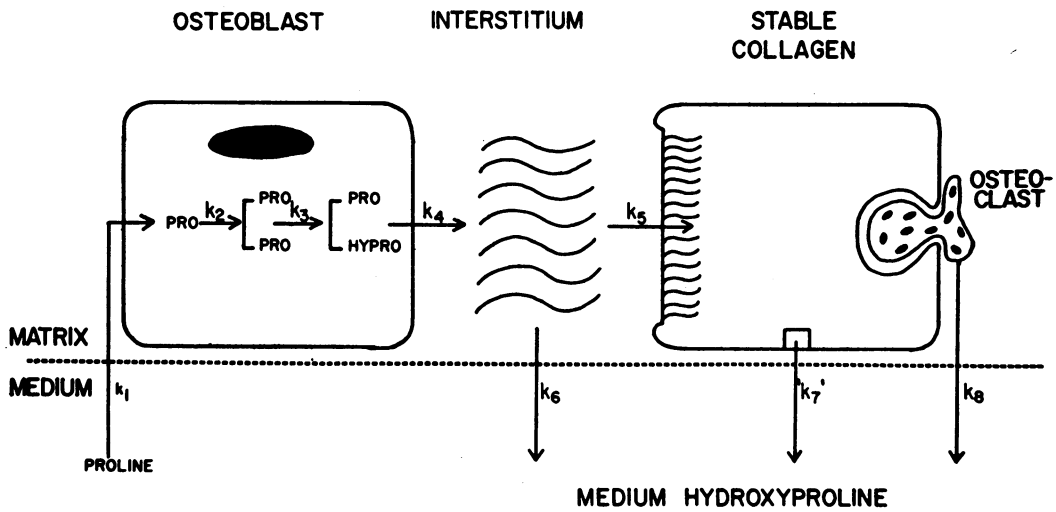

FIGURE 4 A diagrammatic representation of the sources of medium hydroxyproline, and of various rate constants involved. Proline transported into the osteoblast (K_1) is partially transformed into protocollagen (K_2) and precollagen hydroxyproline (K_3) and extruded (K_4) as a soluble hydroxylated polypeptide chain into the interstitium. It is then either deposited (K_5) or escapes into the medium (K_6). "K_7" represents the passive solubilization of matrix collagen occurring during incubation, and K_8, the active cell-mediated resorptive process, is shown here conventionally as being caused by a multinucleate osteoclast.

incubation medium at intervals of 1 hr, for a total of 6 hr. The results are shown in Fig. 5.

The rate of release of labeled hydroxyproline increased up to 3 hr, and remained constant thereafter up to 6 hr of incubation. As it had been shown that the proline inside the cell was fully labeled by 1 hr, it can be concluded that the rate of labeling of the medium after 3 hr may be directly translated into quantities of hydroxyproline synthesized and released into the medium using the specific activity of the medium proline applied.

A complete experiment of this sort is plotted in Fig. 6, where by graphic analysis, K_6, "K_7", and K_8 (See Fig. 4) may be derived. By the extrapolation of the linear portion of the curve of total hydroxyproline release to the ordinate, "K_7" is found. The value in the experiment shown is 1.15 mμmoles/mg collagen and represents a total pool size rather than a rate process, as the release is complete within the time period of the experiment. This quantity of hydroxyproline released passively corresponds to 0.12% of the total collagen of the bone slice.

The slope of the linear portion of the upper curve represents the combined rates of release of newly synthesized collagen (K_6) and cellularly resorbed collagen (K_8). The linear portion of the lower curve represents the rate of accumulation of hydroxyproline counts in the medium after full saturation of the collagen-forming system with proline label. Consequently, a line drawn through zero and parallel with the linear portion of the lower curve represents the total cpm which would have appeared in medium hydroxyproline at 8 hr, if the system were fully labeled at zero time. Using this value (cpm) and the measured specific activity of the initial medium, we may calculate the amount of newly formed collagen accumulating in the medium during the experiment.

The resorption rate can finally be calculated as the difference between the upper linear slope and the lower dotted line; and the rates of new soluble collagen synthesis (K_6) and old collagen resorption (K_8) may then be expressed as true rate processes against time, as both quantities remain linear throughout the incubation period.

Alternatively and more simply, these values can all be derived algebraically, knowing the 3 and 6 hr values for both total hydroxyproline accumulation and total labeled hydroxyproline accumulation. This is possible because, as the graphic plot illustrates, the factors causing deviation from linearity are no longer operative in both instances after 3 hr of incubation. (For elaboration of the algebraic analysis, see Appendix.)

The resorption rate (K_8) derived from the data plotted in Fig. 6 is 0.38 mμmole hydroxyproline/hr per mg collagen. The turnover time for extracellularly deposited bone collagen in vitro on the basis of these values is 54 days, comparing favorably with in vivo data on similarly aged rats where a half-life of 40 days was calculated (23).

Turning to a consideration of the synthesis of matrix collagen which is proceeding simultaneously, Fig. 7 il-

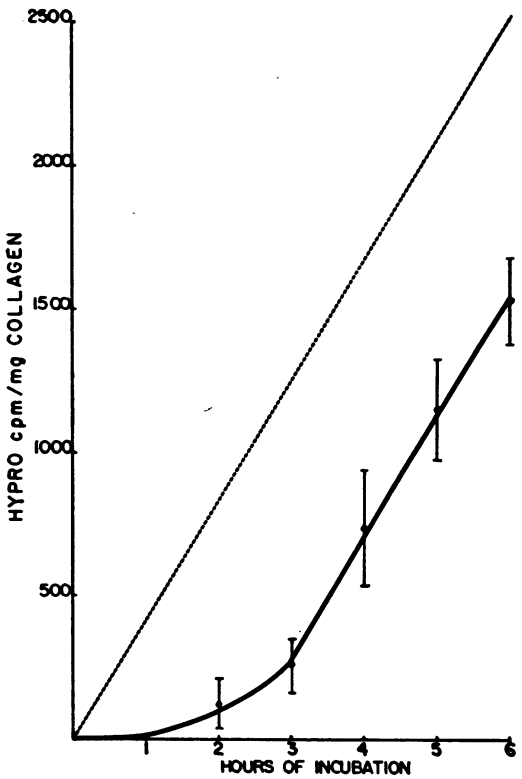

FIGURE 5 The release of labeled hydroxyproline with time from living bone slices incubated at 37.5°C. Following an initial lag period the release rate becomes linear after 3 hr and remains so up to 6 hr. The dotted line represents the theoretical release of labeled hydroxyproline with time which would be expected if the collagen forming system up to and including the final hydroxylated soluble peptide pool were fully saturated with label at zero time. One SEM above and below the line is shown.

lustrates the effect of medium proline concentration on the incorporation of labeled hydroxyproline into the alkali-insoluble collagen of bone. It is clear that the level of external proline concentration determines the degree of labeling up to about 1.0 mM concentration, beyond which point no further increment in labeling occurs up to at least 4.0 mM.

It seems unlikely that the supply of free proline, a nonessential imino acid, would directly limit the synthesis of collagen; we have in fact previously shown directly in this tissue preparation that glucose will provide a source of collagen proline and hydroxyproline (24). The most likely explanation would seem to be that the small intracellular pool of free proline is in effect obliterated at external proline concentrations of 1.0 mM or more. For this reason, all experiments were done at an external proline concentration of 1.5 mM.

We next examined the formation of matrix hydroxyproline against time at this level of external proline. The results are shown in Fig. 8. The formation rate of hydroxyproline which is retained in the tissue is seen to be essentially linear. Although this is theoretically impossible in the presence of any free proline in the bone cell at zero incubation time, the relative amount would appear to be so small compared to the exogenous proline uptake that its retarding effect on the appearance of labeled matrix hydroxyproline cannot be seen. On the other hand, the early delay in the release of hydroxyproline into the medium is seen again in the lower curve.

In the interpretation of these results it is important to recall that matrix incorporation of newly formed hydroxyproline is an expression of the appearance of both intracellular and extracellular labeled hydroxyproline (Fig. 3). The level of applied medium proline was high enough to establish a saturation of the cell proline specific activity throughout incubation. The failure of the matrix labeling to show a deviation from linearity rules out the existence of a significant pool of protocollagen, i.e., nonhydroxylated peptide collagen precursor material.

The presence of a significant pool of hydroxylated collagen peptides would explain the initial lag in the

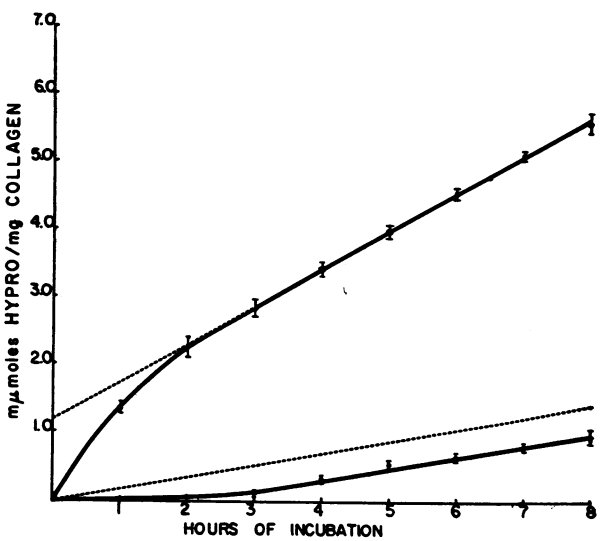

FIGURE 6 Experimental plot to illustrate the three components of medium hydroxyproline. The upper curve shows the cumulative release of hydroxyproline with time. Extrapolation of the linear portion of this curve to the ordinate gives the size of the passively solubilized fraction. The lower curve shows the release of labeled hydroxyproline into the medium. The parallel dotted line represents the true release rate of newly synthesized hydroxyproline. The difference between the slopes of the linear portion of both curves gives the true resorption rate of matrix collagen. Two SEM above and below the upper line, and one SEM above and below the lower line are shown.

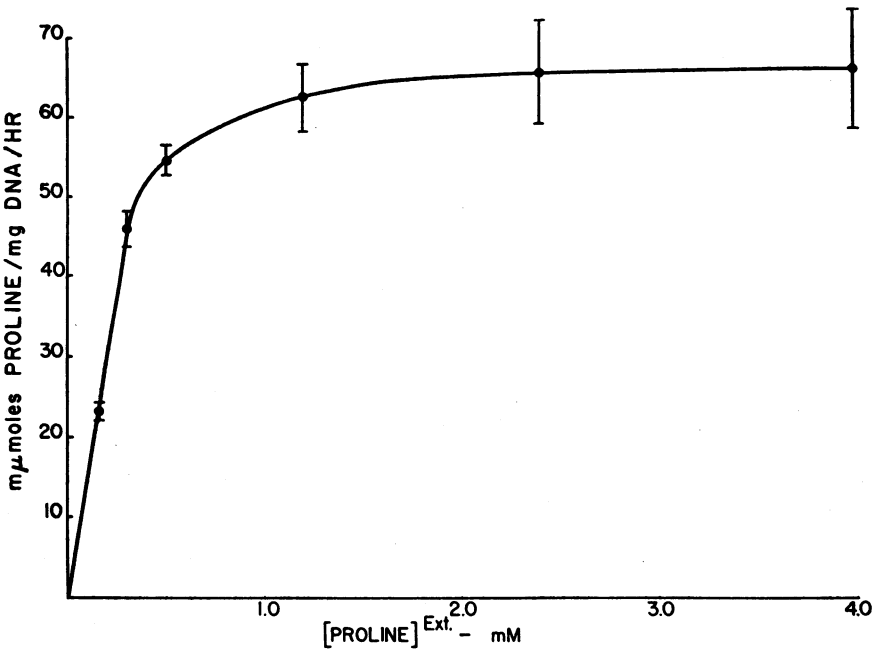

FIGURE 7 The incorporation of labeled proline into a 0.1 N alkali-insoluble collagen fraction of living bone slices in vitro, plotted against external (Ext.) medium proline concentration. Incubation time was 4 hr. Two SEM above and below the line are shown. The external concentration of proline has no effect above the 1.0 mM level.

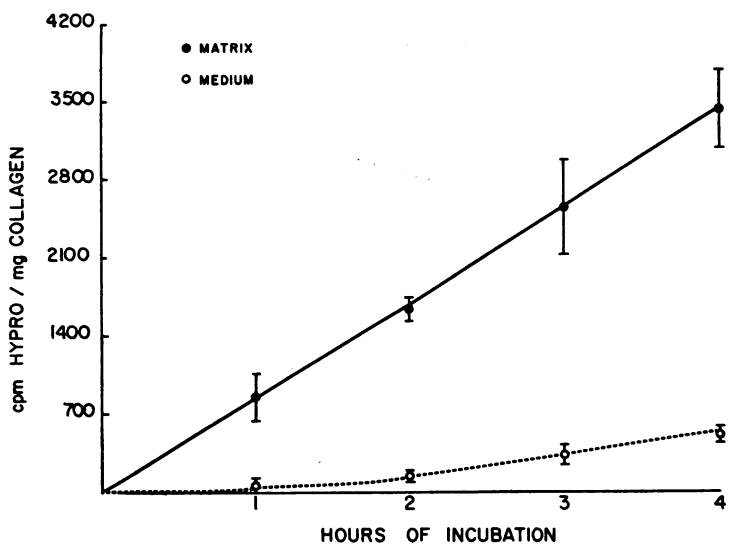

FIGURE 8 The comparative rates of appearance of labeled hydroxyproline in the matrix and in the medium of bone slices incubated in medium containing proline-U-^{14}C. External proline concentration is 1.5 mM. Two SEM above and below the lines are shown. There is no discernible effect on matrix accumulation of new hydroxyproline, whereas there is a distinct lag in the appearance of labeled hydroxyproline in the medium. Better than 80% of the labeled hydroxyproline is retained in the bone matrix.

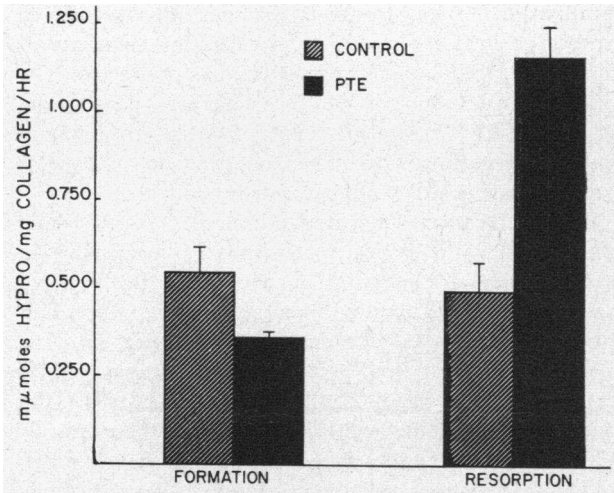

FIGURE 9 The effect of parathormone (Eli Lilly) on bone matrix formation and resorption rates. 21-day old thyroparathyroidectomized animals received subcutaneously 1.0 U/g body weight 18 hr before sacrifice. The means and one SD are shown. Incubation time was 6 hr. There is a clearcut depression of formation rate ($P < 0.01$) and an even more striking elevation of resorption rate ($P < 0.001$). The net effect is a shift from a slightly positive to a grossly negative balance.

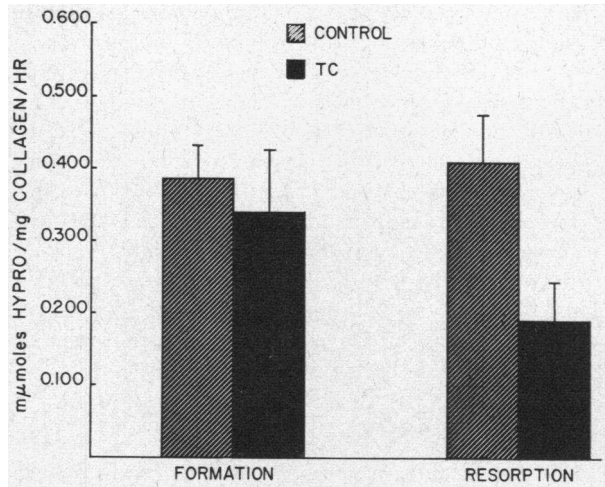

FIGURE 10 The effect of thyrocalcitonin applied in vitro on bone matrix formation and resorption rates. 21-day old thyroparathyroidectomized animals were used. The hormone was applied at a concentration of 10 M.R.C. mU/ml of medium throughout the incubation period. Equal volumes of solvent (0.001 N HCl) were added to the controls. Incubation time was 6 hr. The mean and one SD are shown. There is no significant effect on matrix collagen formation ($P < 0.2$). There is a striking reduction in resorption rate with thyrocalcitonin ($P < 0.01$). The net effect is to convert a slightly negative into a markedly positive balance.

appearance of labeled hydroxyproline in the medium, as newly formed material would have to pass through this pool on its path to final accumulation in the medium.

On the other hand, the matrix hydroxyproline, whether soluble or insoluble, intracellular or extracellular, forms a single pool as measured in these experiments (Fig. 3) and the accumulation of label in this pool cannot deviate from linearity as long as the specific activity of the protocollagen pool remained stable, and as long as the rates of all processes remain linear.

It may be noted that approximately 80% of the newly synthesized collagen appears to be retained in the tissue while about 20% is either not deposited or is immediately released.

The effects of parathyroid hormone given in vivo on the system were next studied. 21-day old animals were used 24 hr after thyroparathyroidectomy to eliminate the possible effects of thyroid hormone deficiency on the skeletal responses. The absence of endogenous parathyroid hormone and thyrocalcitonin allowed evaluation of the effects of the single dose of exogenous hormone given 18 hr before sacrifice. The results obtained are shown in Fig. 9 and in Table I.

Fig. 9 shows the development of a clear-cut and significant fall in formation rate and an even more striking increase in resorption rate. The net result is the production of a strongly negative collagen balance.

TABLE I

Effects of PTE and Thyrocalcitonin on the Mean Solubility of Bone Matrix Collagen and the Mean Deposition Ratio of Newly Formed Collagen

	Mean collagen solubility	SD	No. of samples	P	Mean deposition ratio	SD	No. of samples	P
	%				%			
Control	0.295	±0.100	6	—	57.0	±4.5	6	—
+ Parathyroid	0.103	±0.074	6	<0.01	49.7	±3.5	6	<0.01
+ Thyrocalcitonin	0.222	±0.073	6	<0.70	58.0	±5.0	6	<0.8

Bone taken from 21-day old thyroparathyroidectomized animals 24 hr after surgery. Hormone dosage and units of administration as described above. The *P* values represent the differences of the treated groups from the control group. Thyrocalcitonin had no significant effect on either quantity. Parathyroid hormone treatment induced a significant depression of the soluble pool of matrix collagen, and of the ratio of newly formed collagen which is retained in the tissue.

Table I shows the changes which are found in collagen solubility and deposition ratio. There is a diminution in the size of the soluble collagen pool after parathyroid treatment, and there is also a small but significant decrease in the deposition ratio of newly formed collagen.

The response of the system to thyrocalcitonin in vitro is shown in Fig. 10 and Table I. There is no significant effect on the formation rate of collagen, but there is a striking reduction in the resorption rate. The net effect is the production of a markedly positive collagen balance. Table I reveals that these changes are unaccompanied by any effects either on collagen solubility or on the deposition ratio.

DISCUSSION

It is clear from these experiments that the measurement of total hydroxyproline released by a bone tissue slice in vitro is not a measure of the turnover rate of collagen, but rather presents a composite picture of three differing phenomena. The necessity of disentangling the contributions of synthesis, resorption, and passive solubility, while time consuming, is of considerable value since it allows a fairly meaningless single value to be resolved into three components, all potentially valuable in understanding collagen metabolism in bone.

The validity of identifying K_8 as the resorption rate is confirmed by its appropriate response to parathyroid hormone and thyrocalcitonin. Many workers using bone slices in vitro or in tissue culture have confirmed the direction of these hormonal responses with indexes of both matrix and mineral deposition and renewal (7, 25–28). This system, however, presents several advantages over previous methods. The quantitation is sufficiently precise to allow comparison of the formation and resorption rates and therefore of collagen balance in vitro. In addition, the dimensions of the soluble tissue collagen pool ("K_7") can be measured, as can the ratio of newly formed collagen which is retained in matrix to the total collagen synthesized, a quantity to which we have given the name the deposition ratio. Both of these parameters yield important information about bone collagen metabolism.

Prior studies of matrix metabolism have been based either on the release of total hydroxyproline into the medium or on the release of counts from prelabeled matrix collagen. No previous attempts have been made to distinguish between the contribution of these three processes to the medium hydroxyproline.

As we have seen both matrix solubility and the deposition ratio of newly formed collagen can be altered by parathyroid hormone treatment. These parameters are also affected by other factors including the ageing process (29).

The reduction in deposition ratio under the influence of parathyroid hormone suggests that not only is collagen synthesis retarded but also the organization and maturation of extracellular collagen is obviously affected. The reduction in the pool of soluble collagen by parathyroid extract (PTE) treatment and not by thyrocalcitonin suggests that the small soluble collagen pool of bone is primarily composed of immature new collagen.

The significance of newly synthesized collagen which is not laid down is unclear, but it is by no means certain that it can be attributed to an artifact of the in vitro system. Rapidly turning over pools of collagen, including bone collagen, have been described by others in vivo (23, 30). Although some recent evidence suggests that the bone cells may be more directly concerned (31), the process of deposition and organization of the extracellular matrix is generally thought of as a series of physicochemical reactions and the ordering of these reactions represents an area of considerable ignorance (32). Moreover, it seems not unlikely that such a process might contain within it a degree of inherent inefficiency.

The usefulness of the kind of information supplied by this approach lies in its direct application to human bone studies. We have previously shown that human bone removed at biopsy is qualitatively similar to animal bone in terms of its major biochemical characteristics, and that it is amenable to comparative quantitative study (33). Clear-cut changes may be demonstrated in human hyperparathyroidism (34), but the picture so far has proved confused in osteoporosis where changes in matrix formation rate both directions from normal have been observed. No method was available at that time to evaluate the rates of collagen deposition and resorption (35). The increased precision supplied by these methods will permit the rates of both matrix formation and breakdown and consequently matrix balance to be measured in a single biopsy sample in any given metabolic disease.

The advantages of direct measurements of the resorption rate of collagen in terms of reproducibility, simplicity, and precision over the tedious combination of mineral balance studies and isotope dilution curves with all their uncertainties is clear. Indeed, the uncertainty shifts to the question of the validity of extrapolating from in vitro to in vivo and from a single sample to the entire skeleton.

Using this approach, it is now possible to correlate the actual resorption rate and other measurable quantities which may determine the cellular bone potential for resorption, such as lysosomal enzyme content (36) and collagenolytic activity (37). The latter has already been shown to change in parallel with the over-all hydroxyproline release rate with age (22).

APPENDIX

The calculation of the actual values of the rate process K_5, K_6, and K_8 and of the pool size "K_7" may be done alge-

braically. Consideration of Fig. 8 shows that the linear portion of the curves of total and labeled hydroxyproline (hypro) release may be measured by collection of media over any time period from 3 to 8 hr of incubation which will yield enough labeled and total hydroxyproline for accurate quantitation.

For convenience we have usually collected the 1st, 2nd, and 3rd hour media into a final single pool and the 4th, 5th, and 6th hour media into a second final pool before hydrolysis and estimated the total labeled and unlabeled hydroxyproline in these two timed collections.

Using these values the following formulae may be developed:

A. Solubility % (mμmoles hydroxyproline passively released expressed as a percentage of the total mμmoles hydroxyproline in the tissue)

$$= \frac{[\text{total 6 hr hypro released in m}\mu\text{moles} - 2(\text{3–6 hr hypro released) in m}\mu\text{moles}] \times 100}{\text{total m}\mu\text{moles tissue hydroxyproline}}$$

B. (Resorption rate of hypro + synthetic rate of medium-soluble hypro) in mμmoles/hr
= 1/3(3–6 hr hypro released into medium)

C. (Synthetic rate of medium-soluble hypro) in mμmoles/hr
= 1/3 · (cpm hypro in 3–6 hr medium)/
(initial medium proline SA in cpm/mμmoles)

D. Resorption rate in mμmoles/hr = B − C

E. Matrix hydroxyproline deposition rate in mμmoles/hr
= 1/6 · (matrix hypro cpm after 6 hr)/
(initial medium proline SA in cpm/mμmoles)

F. Total hydroxyproline synthesis (mμmoles)/hr = E + C

G. Deposition ratio $= \frac{E}{F} \times 100$

All of the rate processes may be referred to either a tissue DNA or tissue collagen base. In this paper a tissue collagen base is used throughout. The quantity A (solubility) is an exception. It represents a pool size rather than a rate process, and must always be referred to the tissue hydroxyproline (collagen) content. The deposition ratio is obviously a unit-free factor and measures the tendency of newly formed collagen to be incorporated into the solid phase.

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