CHANGES IN URINARY HYDROXYPROLINE BY FIVE HEALTHY YOUNG ADULT MALES DURING AMBULATION AND HORIZONTAL BED REST RECUMBENCY

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We hereby recommend that the thesis prepared under our supervision by Patricia Moody Snyder

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INTRODUCTION

Integration of nutrition into the health support system of the United States space program did not become critically necessary until the Gemini IV flight of McDivitt and White in June 1965. All work in the space program directed at supplying adequate support for the health of astronauts requires a team effort of professionals of varied background. They apply a thoroughly integrated approach to the common problem. This means that physicians, physicists, engineers, nutritionists, and research workers in many fields must combine their efforts, each as a part of a whole system. This is what is meant when one speaks of the "system approach."

Astronauts are not controllable hospital patients. They are, on the contrary, magnificently healthy men doing a highly complex job, and this job has made it impossible to allow them to serve as biological test subjects. Instead, bed rest subjects must serve as substitutes, to aid in learning as much as possible about metabolic processes during inactivity.

Because of the author's interest in the turnover of collagen in bone, she has selected for her master's research problem the part of the current study which relates to the excretion of hydroxyproline, which is

found almost exclusively in collagen. The investigation was made during a series of Periods in a study which involved two Bed Rest Units with related ambulatory periods.

The present study incorporates the use of Ca^{47} in conjunction with immobilization. The changes in the excretion of hydroxyproline in the urine were studied in five men during bed rest and ambulation as described in this report. This investigation was conducted in the Nelda Childers Stark Laboratory for Human Nutrition Research in the Texas Woman's University Research Institute. Blood, urine and feces samples were analyzed during the study as a means of detecting metabolic changes in healthy subjects.

The author used the Prockop-Udenfriend Method (61) for the measurements of urinary hydroxyproline in this study. This measurement is carried out by the imino acid hydroxyproline being oxidized to Δ'-pyrroline-4-hydroxy-2-carboxylic acid and pyrrole-2-carboxylic acid, then with the formation of a chromophore with p-dimethylaminobenzaldehyde. This investigation involves a number of reagents and biochemical techniques (62).

The specific objectives of the author's study have been the following:

1. To make daily urinary analyses throughout the entire study and to find the quantity of hydroxyproline excreted by five men during the following periods: (a) a twenty-nine day Pre-Bed Rest Ambulatory Period; (b) a 14-day First Bed Rest Study, during which 800 mg. of calcium were fed daily; (c) a fourteen-day Interim Ambulatory Period; (d) a fourteen-day Second Bed Rest Study during which 300 mg. of calcium were fed daily; and (e) a fourteen-day Post-Bed Rest Ambulatory Period.

2. To find possible statistical relationships during the various ambulatory and immobilization periods between the level of urinary hydroxyproline excreted and the level of calcium intake.

REVIEW OF LITERATURE

L-Proline and 4-hydroxy-L-proline are major building stones of collagen, the protein of connective tissue which constitutes one-third of the total body protein. While proline occurs in all proteins, trans-4-hydroxy-l-proline, together with a small amount of trans-3-hydroxy-L-proline, generally is found only in collagen. Because of the accurate and facile analytical methods for detection, hydroxyproline offers a convenient handle for following biosynthesis and degradation of collagen (55).

Collagen is one of the scleroproteins, and it accounts for about 30 per cent of the body proteins. It exerts an architectural function throughout the body, most notably in skin, tendons, cartilage, blood vessels; connective tissue, organ capsules, and bone matrix. It confers the ultimate in "togetherness" and is responsible for making one an "intact" individual (68).

Collagen not only is the most abundant, but one of the most unusual animal proteins, since it is devoid of cysteine and tryptophan and contains more than 30 per cent glycine and such unusual imino acids as hydroxyproline and hydroxylysine. In animal tissues hydroxyproline and hydroxylysine are essentially found only in collagen. The formation of

collagen therefore is of interest not only as a problem in protein biosynthesis, but also as one in the biosynthesis of these unusual imino acids (67).

Since hydroxyproline is found almost exclusively in collagen, the determination of this imino acid has been utilized for the estimation of collagen in various tissues. Collagen has been considered to be rather inert metabolically, although bone, liver and periodontal membrane exhibit an especially rapid turnover of this substance (17).

The first biological material from man studied extensively for hydroxyproline content was urine. Total urinary hydroxyproline consists of one to three per cent of free hydroxyproline and more than 95 per cent in a peptide-bound form. Fourteen different hydroxyproline peptides have now been isolated and identified in human urine. The dipeptide, prolyl-hydroxy-proline comprises about 60 per cent, and the tripeptide, glycyl-prolyl-hydroxyproline about 15 per cent of the hydroxyproline peptides excreted. Thus the amino acid composition and sequences of seventy-five of isolated urinary peptides are identical with known sequences in collagen. The composition of 10 of the remaining 12 peptide also is consistent with their origin from collagen (59).

OCCURRENCE

Hydroxy-L-proline was discovered in gelatin hydrolysate in 1902 (21). Apart from its well-known presence in gelatins and collagens (25) (26), it occurs bound in alfalfa protein (70), sugar-beet protein (65), Sacrina lutea (6), dentin protein (53), horse-radish peroxidase (54), proteins of insect cuticle (28), and in the antibiotic actinomycin Xo beta (8) (7).

Hydroxy-L-proline occurs in the free state in pollen (3), prunes (35), the hemolymph of Drososophila melanogaster (2), the sporulation medium of Bacillus globiggi (14), and the blood and malpighian tubes of the larvae of Bombyx mori infected with polyhedral disease (15).

NORMAL METABOLISM

When Lindstedt and Prockop (52) followed the specific activity of urinary hydroxyproline after the injection of radioactive proline into young rats, they found evidence suggestive of at least three different pools of hydroxyproline, with half-lives of one, five, and 50 to 100 days. The pool with the long half-life was thought to present insoluble collagen. The other pools were considered to represent the metabolically active soluble collagens which contribute most to urine hydroxyproline. More recently Prockop has presented evidence that insoluble

collagen may also contribute significantly to the urinary hydroxyproline peptides (59).

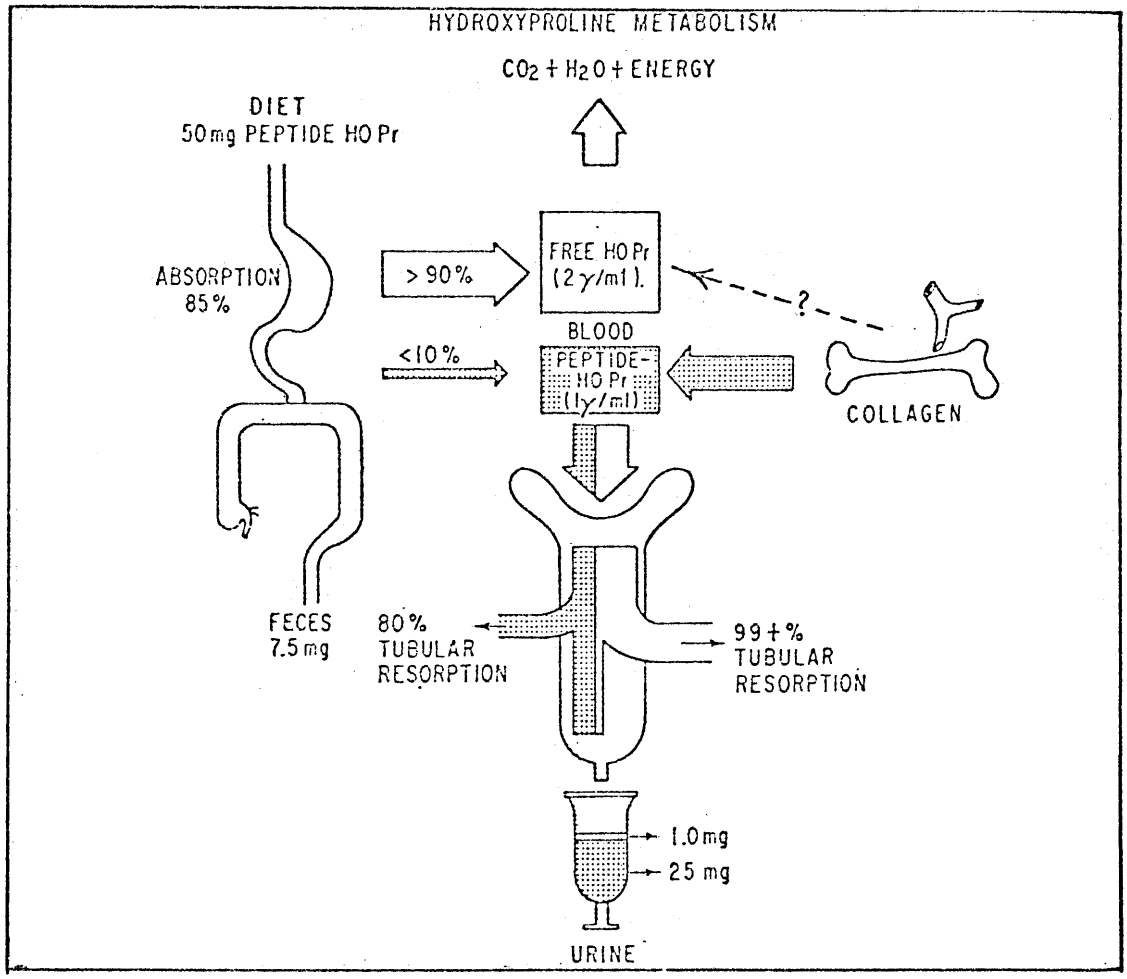
Calculations based on the urinary excretion indicate that at least seven to eight per cent of the hydroxyproline ingested as gelatin is absorbed in the peptide form, with these peptides rapidly cleared by the kidney.

A gelatin-free diet contains about 50 milligrams of hydroxyproline, all in a bound form, either as protein or peptide.

In metabolic balance studies it was found that 85 per cent undergoes intestinal absorption and that about 7.5 milligrams/day is recovered in the feces. Of the hydroxyproline that is absorbed, more than 90 per cent is the free amino acid. The blood plasma contains free, peptide-bound, and protein-bound hydroxyproline. Most of the free hydroxyproline is metabolized rapidly to carbon dioxide and water. The major source of peptide-bound hydroxyproline in blood is thought to be soluble collagen in bone, skin, tendons, and other collagen depots.

Both free and peptide hydroxyproline are filtered by the glomeruls. The free amino acid undergoes 99 per cent tubular resorption; and less than one milligram/day appears in the urine. Peptide-bound hydroxyproline is only eight per cent reabsorbed and about 25 milligrams/day is excreted. The renal clearance of peptide hydroxyproline at high

plasma levels approaches the glomerular filtration rate. Thus, one must be certain that the patient's renal function is normal if his urinary hydroxyproline is to be compared with that of normal subjects. In a study of four patients with severe renal impairment, all showed elevated plasma levels and decreased urine levels of peptide-bound hydroxyproline. The overall metabolism of hydroxyproline in man is shown in Figure 1.



According to Darwin Prockop (59)

FIGURE 1. SCHEME OF HYDROXYPROLINE METABOLISM IN A NORMAL ADULT SUBJECT

Actively growing subjects have a much higher hydroxyproline excretion than nongrowing subjects. This is more marked during the teen years when the adolescent growth spurt occurs. There is no significant change in hydroxyproline excretion with advancing age once growth has ceased. The mean normal value of 26.3 milligrams/day is the average urinary hydroxyproline excretion for the 40 normal subjects who reported no linear growth during the preceding year. The normal range defined as two standard deviations from the mean is 13.5 and 39.1 milligrams/day (68).

The magnitude of the daily excretion of urinary peptides and their variety in normal individuals make them extremely interesting as indices of the protein metabolism, which they must reflect. The present consideration of the small minority of these peptides that contain hydroxyproline, about 5 per cent of the total by weight, is based on the fact that this imino acid pinpoints their origin to collagen, a protein which comprises about one-third of the protein of the body and contains 13 per cent hydroxyproline. Though hydroxyproline is present in other substances of the body, such as brain lipoprotein, only collagen and elastin have been shown to contain significant amounts. The relatively much smaller amount of elastin in the body and its low hydroxyproline content of 1.5 per cent make it quantitatively a less likely precursor of the urinary hydroxyproline peptides than collagen (71).

It is possible that the decreases in soluble collagen content and urinary hydroxyproline peptides are independent reflections of an overall decrease in collagen metabolism with age and that they are not directly related. Since 40 per cent of body collagen is in the matrix of bone, its metabolism in this tissue has attracted much recent investigation. Bone collagen has been found to turn over more rapidly than collagen in other tissues, and it has been suggested (18) that bone provides a reservoir of mature collagen, the catabolism of which gives rise to most of the urinary hydroxyproline peptide excretion. Evidence that mature collagen provides a significant contribution to urinary hydroxyproline already has been presented. The reorganization of bone structure which occurs during growth might in fact provide an area of continuous breakdown and resynthesis of collagen (71).

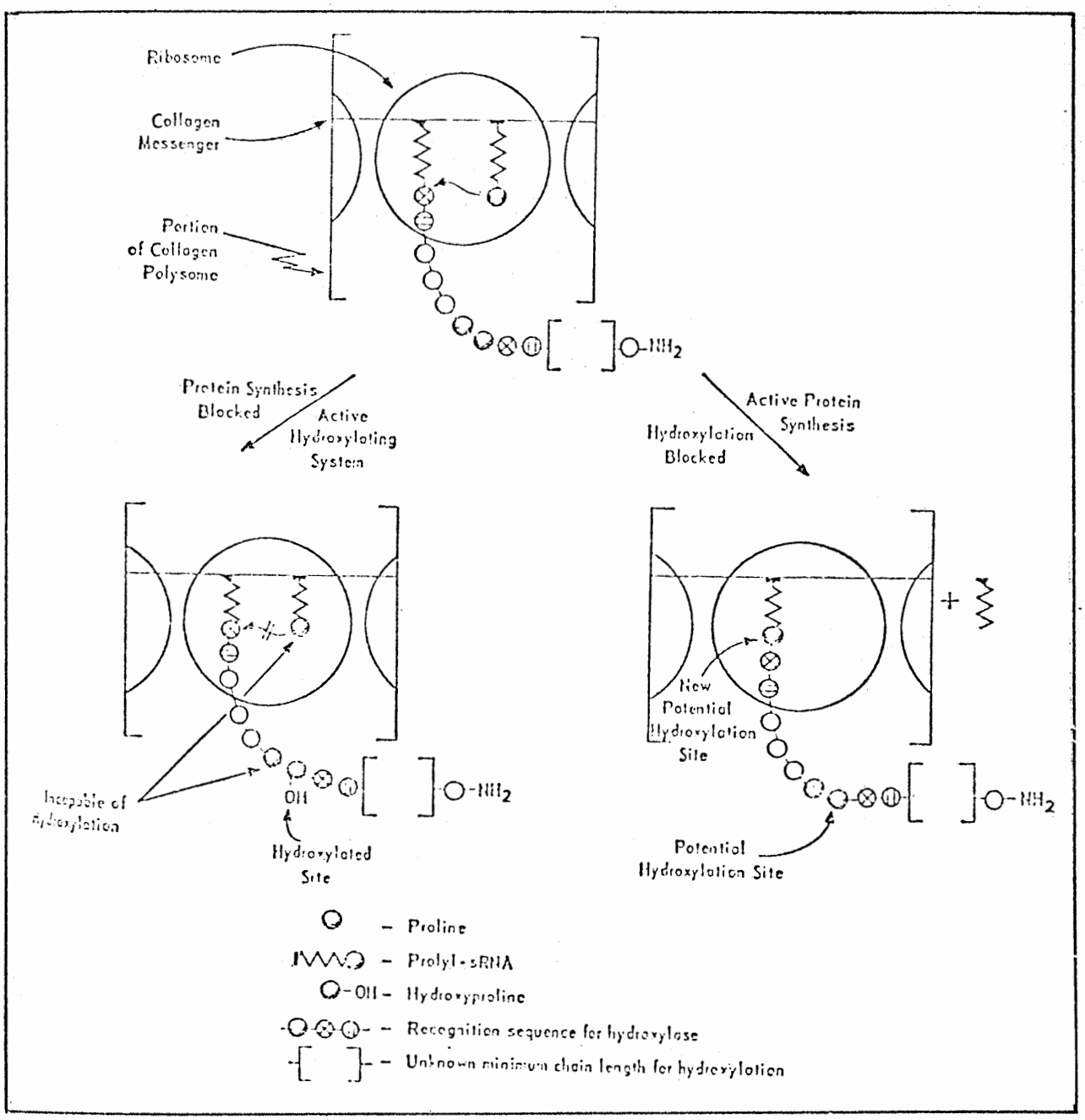
HYDROXYLATION OF PROLINE DURING COLLAGEN BIOSYNTHESIS

Proline is activated and converted into prolys-sRNA, which then attaches itself to the ribosome and is incorporated into a ribosomal bound peptide. When the ribosomal-bound peptide reaches a definite size and contains appropriate sequences which can be recognized by the hydrolylase, certain proline residues are hydroxylated to hydroxyproline residues. In the absence of oxygen, however, ascorbic acid or the hydroxylating enzyme the ribosomes form a proline -rich, hydroxyproline-deficient

protein. In the chick embryo system this protein can be hydroxylated by subsequent introduction of appropriate conditions for hydroxylation.

Apparently the hydroxyproline-deficient protein can be hydroxylated even after separation from the ribosome and after the addition of inhibitors of protein synthesis. Furthermore, some of the prolines in synthetic polymers such as $(pro\text{-}gly\text{-}pro)_n$ also can be hydroxylated. It would appear, therefore, that the substrate specificity of proline hydroxylase is to a large extent determined by a peptide sequence. Conceivably the attachment to the ribosome may have some additional effect on the rate or site of hydroxylation. This can be explained by a mechanism which is shown diagrammatically in Figure 2.

Since lysine is no doubt hydrolylated by a comparable mechanism, a generalized scheme for hydroxylation may be considered. The ribosome accepts amino acyl sRNA molecules destined for collagen. As polymerization proceeds, two conditions begin to be met which allow proline and lysine residues in peptide linkage to become substrates for the hydroxylase: (a) the peptide grows to a minimum size, and (b) the two amino acids become incorporated into definite sequences which can be recognized by the specific hydroxylases. In the presence of sufficient hydroxylating enzymes and cofactors, hydroxylation keeps pace with peptide synthesis so that when the protein chain is completed it is fully hydroxylated. Under such conditions hydroxylation occurs during the



According to Albert Sjoerdsma and co-workers (67)

FIGURE 2. SCHEME FOR THE HYDROXYLATION OF PROLINE DURING COLLAGEN BIOSYNTHESIS

process of translation. When hydroxylation is limited, ribosomal peptide-synthesizing mechanisms are, for a time at least, unaffected; and they continue to elaborate the usual chain, which is, however, deficient in or devoid of hydroxylated residues. No large quantities of unhydroxylated "collagen" have been accumulated experimentally. It should be noted that collagen is composed of two different peptide chains and that each contains hydroxylated amino acids. The same enzyme may be responsible for hydroxylation of both chains.

The hydroxylation of an sRNA amino acid is a process as complex as the hydroxylation of an amino acid in peptide linkage (67).

CORRELATION OF URINARY HYDROXYPROLINE, SERUM ALKALINE PHOSPHATASE AND SKELETAL CALCIUM TURNOVER

Thirty-three patients underwent 70 simultaneous calcium^{47} kinetic changes, together with calcium, phosphorus and nitrogen metabolic balance studies. Concomitantly, total urinary hydroxyproline and serum alkaline phosphatase were determined. The group was weighted in favor of patients with osteoporosis, vitamin D-resistant rickets, metastatic bone disease and parathyroid disorders. Total urinary hydroxyproline, serum alkaline phosphatase, calcium accretion rate and calcium resorption rate correlate well among the metabolic bone disease groups. There is a good correlation in a given individual between serum alkaline phosphatase and calcium accretion rate, and between urinary

hydroxyproline and calcium resorption rate. The combination of serum alkaline phosphatase and urinary hydroxyproline is a useful screening method for the detection of skeletal diseases with high turnover rates.

Elevated urinary hydroxyproline was associated with high calcium resorption rates with only four exceptions. Furthermore, the majority of patients with low urinary hydroxyproline (mostly osteoporotic cases) had low calcium resorption rates. However, six out of 38 subjects with high calcium resorption rates had normal urinary hydroxyproline levels.

Notable exceptions to the correlation of urinary hydroxyproline with the accretion rate or serum alkaline phosphatase include the single study of W. S., a patient with a hyperparathyroid-like state secondary to widespread pancreatic carcinoma, but with only one small bony metastasis, the third study of B. C., a patient with vitamin D-resistant rickets under treatment with large doses of vitamin D for five months, and the single study of M. S., a patient with severe hyperthyroidism. Patients with markedly negative calcium balances (greater than 250 mg./day), H. L., W. S., M. G. and M.S., showed high urinary hydroxyproline excretions regardless of the presence or absence of an elevated accretion rate or serum alkaline phosphatase. H. L. and M. S. had an equally high resorption rate but there was a two-fold difference in their urinary hydroxyproline levels. Three of the above four patients had carcinoma with marked hypercalcemia.

The correlation of the changes in urinary hydroxyproline, serum alkaline phosphatase, calcium accretion and calcium resorption in 46 sequential studies in 22 patients also was analyzed to evaluate the urinary hydroxyproline and serum alkaline phosphatase as indices of changes in bone formation or bone resorption with treatment. Changes in serum alkaline phosphatase gave a 0.30 coefficient of correlation with changes in calcium accretion; but there was a random association between changes in urinary hydroxyproline and changes in serum alkaline phosphatase, calcium accretion and calcium resorption.

Total urinary hydroxyproline, serum alkaline phosphatase and skeletal calcium turnover correlate well when metabolic disease groups are compared. Furthermore, the calcium resorption rate and to a lesser degree the calcium accretion rate have shown a correlation. Changes in serum alkaline phosphatase hydroxyproline are less sensitive than radiocalcium kinetic studies as an index of an effect of treatment on skeletal mineral turnover (48).

The general correlation of growth (33) and serum alkaline phosphatase (43) (45) (46), with urinary hydroxyproline is consistent with the former explanation. The increased levels of urinary hydroxyproline in destructive bone diseases such as Paget's disease, hyperparathyroidism, metastatic bone disease and after administration of parathyroid extract

has been used as supportive evidence for the latter hypothesis (17) (36). The data presented do not conclusively support either hypothesis.

It generally is concluded that the major fraction of the serum alkaline phosphatase originates from osteoblasts and chondroblasts, while a small fraction may be produced by liver cells (27) (9). Although kidneys, intestinal mucosa brain and leukocytes are rich in alkaline phosphatase, only in diseases of the skeleton and liver are there appreciable elevations of serum alkaline phosphatase (13). The precise role of alkaline phosphatase in bone formation remains unclear, although it may participate in calcification on an extracellular basis since calcification does not appear within the osteoblast as defined by light microscopy. Irving (32) has summarized evidence showing that alkaline phosphatase is associated with bone matrix production rather than with the subsequent calcification.

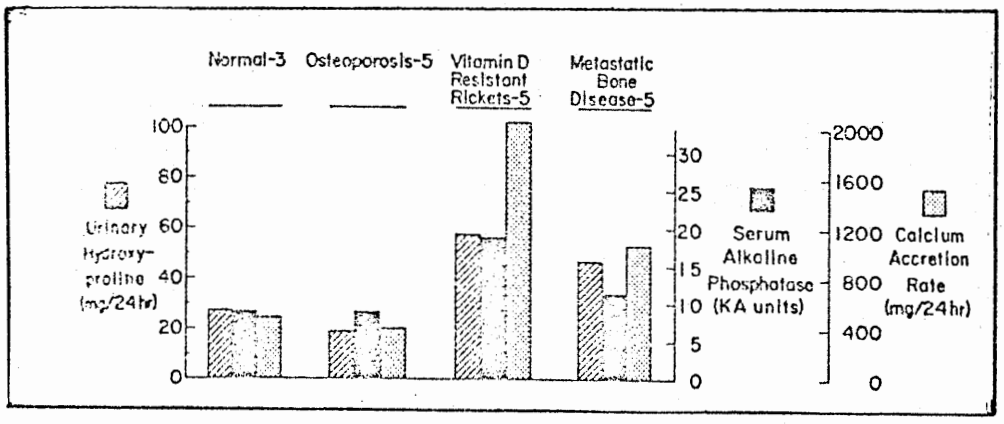
Considerable indirect evidence supports that the serum alkaline phosphatase as an index of the rate of bone formation. The near perfect correlation between the increment of height and serum alkaline phosphatase from infancy to adulthood as shown by Clark and Beck (11) is consistent with serum alkaline phosphatase being a reflection of bone formation. The presence of a high serum alkaline phosphatase in disease states characterized by increased bone formation, including Paget's disease, osteoblastic metastatic bone disease and osteogenic sarcoma,

and of a low serum alkaline phosphatase in disease states characterized by decreased bone formation such as scurvy, creatinism and hypophosphatasia adds further evidence that the serum level reflects the rate of bone formation. In most of these states there is a general correlation between serum alkaline phosphatase and urinary hydroxyproline (43).

Considerable debate still continues as to the validity of the calcium accretion rate as an index of bone formation. The main issue relates to what fraction of the accretion rate reflects the movement of radiocalcium from the rapidly miscible calcium since even true bidirectional exchange will initially appear unidirectional when "it occurs between a small active compartment and a large sluggish one" (29). Heaney and Whedon (30) have suggested that the fraction of the accretion rate due to inert exchange is less than 25 per cent based on the rise of the accretion rate from 308 to 145 mg./day after prolonged hormonal therapy in (J. W.) a patient with senile osteoporosis, indicates that the fraction of the accretion rate due to inert exchange is at least less than 50 per cent. Furthermore, the presence of positive calcium balances, 50 to 100 mg. in excess of accretion rates as seen in the final two studies of D. N. (osteoporosis) and in the single study of T. R. (Laennec's cirrhosis) adds further evidence that accretion rates are not falsely high due to inert exchange since calcium retention cannot exceed the rate of bone formation (in the absence of widespread soft tissue calcification) even if resorption is at a standstill. Bidirectional exchange has

no effect on calcium balance. Finally, the correlation of serum alkaline phosphatase and accretion rate supports the validity of the latter as an index of bone formation.

A good correlation between the total urinary hydroxyproline, serum alkaline phosphatase, calcium accretion rate and calcium resorption rate was observed among the mean values of the control studies for each major disease group (Figure 3). A moderate reduction in both urinary hydroxyproline and calcium turnover rate was noted in a majority of subjects with senile and postmenopausal osteoporosis, but the mean serum alkaline phosphatase was normal. Both the groups with vitamin D-resistant rickets and metastatic bone disease showed roughly proportional elevations of the four parameters except that the calcium accretion and resorption rates were disproportionately higher than the urinary hydroxyproline or serum alkaline phosphatase among subjects with resistant rickets (48).



According to LeRoy Klein and co-workers (48)

FIGURE 3. COMPARISON OF MEAN URINARY HYDROXYPROLINE, SERUM ALKALINE PHOSPHATASE, AND CALCIUM ACCRETION RATE AMONG NORMAL SUBJECTS AND THREE SKELETAL DISEASE GROUPS. THE MEAN CALCIUM RESORPTION (NOT SHOWN IN FIGURE) APPROXIMATED THE MEAN CALCIUM ACCRETION IN EACH GROUP.

GROWTH HORMONE

Human growth hormone increases urinary hydroxyproline. The high excretion of hydroxyproline in children is consistent with the general view that bone formation and resorption rates are greater in children than in adults (17).

Growing animals and guinea pigs with osteolathyrism have increased amounts of tissue-soluble collagen and increased urinary hydroxyproline levels. On the other hand, severe dietary restriction of protein in man and in guinea pigs fails to lower the urinary hydroxyproline

excretion despite expected decrements in overall protein synthesis, including the newly formed soluble collagen.

Complete balance studies and blood-disappearance curves after administration of radioactive calcium and strontium have provided useful indices of bone mineral metabolism and net calcium balance in the past. The present data suggest that urinary hydroxyproline may be an equally useful indicator of bone collagen metabolism. The rapid excretion of hydroxyproline after the administration of hormones known to alter bone metabolism and the minimal bried dietary requirements of such studies suggest that urinary hydroxyproline measurements may provide a practical clinical guide to response to growth-hormone treatment and a rapid and useful index to the metabolic activity of bone matrix in various diseases (17).

More detailed study of the levels of excretion of hydroxyproline in relation to growth has been published by Jasin, Fink, Wise, and Ziff (33). They showed that urinary hydroxyproline is increased in growing children and in patients with acromegaly, with decreases when growth stops, as in adults, treated acromegalics, and patients with dwarfism. Gross (23) has shown that the amount of soluble collagen in the skin of guinea pigs is proportional to their growth with decreases whenever growth ceases. Growth hormone has been shown to stimulate protein synthesis at a subcellular level and to cause collagen deposition in

growing subjects and in patients with acromegaly. Thus, changes in urinary hydroxyproline induced by growth hormone would seem to indicate primarily stimulation of collagen synthesis.

The only young patient excreting less than normal amounts of hydroxyproline, in this case, 9.4 milligrams/day, was an eight-year-old girl with untreated myxedema, due to an inability of the thyroid gland to trap iodine. She had had no linear growth for several months up to the time of the urine assays. When thyroid hormone was administered in sufficient amounts to make her euthyroid, urinary hydroxyproline rose to 145 milligrams/day and significant growth was observed (68).

The daily urinary excretion of hydroxyproline has shown a high positive correlation with growth rates and has gained significance as an index of collagen metabolism. Infants, children, and adolescents undergoing rapid growth excrete more hydroxyproline than do nongrowing healthy adults.

Non-healthy full term infants, from birth to nine weeks of age, were studied sequentially with regard to their daily urinary output of free and total hydroxyproline, creatinine, phosphorus, and calcium. Both free and peptide hydroxyproline excretion were low at birth and rose rapidly during the first 10 to 14 days of life. The very low excretion rate of hydroxyproline noted at birth and the subsequent rapid

increase were attributed to changing growth rate as well as alterations in renal function (49).

A series of investigations was conducted to evaluate the influence of exogenous growth hormone on the growth and metabolism of premature infants. Knowledge in this field is very scanty. Grunbach and Ducharme (16) have reported suggestive data for a lack of response of premature bone tissue to somatotrophic hormone (STH) stimulus. This finding, at least in part, is in contradiction to the possibility of increasing the size of animal fetuses by injecting pregnant mothers with STH, as has been shown in the past by some authors. Moreover, almost nothing is known concerning the influence of STH on nitrogen, calcium, and phosphorus metabolism very early in life. The only paper on humans contributing to this subject has been recently published by Vest and co-workers (72). Metabolic balance data, however, have been reported only for one 28-day-old infant for whom the STH treatment was given for a very short period. It was not possible to register any increase of linear bone growth after hormone administration. Concomitantly urinary hydroxyproline excretion did not change significantly.

Nitrogen balance studies indicated a sharp increase of nitrogen retention, due to a reduced urinary excretion, in all infants. Calcium and phosphorus balances rose in three out of four premature infants treated with growth hormone, but the characteristic STH calciuric action

of STH was not observed. Furthermore, STH failed to induce any significant increase in non-esterified fatty acid serum concentration of premature infants.

It therefore may be concluded that the metabolic response of premature infants to STH differs consistently from that normally observed in more mature subjects (10).

VARIOUS DISEASES

The values of urinary free and peptide-bound hydroxyproline were measured in 72 control subjects and 89 patients with various diseases, and the values of serum free hydroxyproline in 95 control subjects and in 86 patients. Age, sex and body size were found to influence the values in the control subjects. For clinical purposes the following normal values are suggested: urinary total hydroxyproline for persons 18-21 years old 13.0 - 28.0 mg./24 hours and for persons 22-55 years old 8.5 - 23.5 mg./24 hours; serum free hydroxyproline for males 0.70 - 1.55 γ/ml. and for females 0.70 - 1.40 γ/ml. Increased values occurred in hyperthyroidism, acromegaly, Turner's syndrome, hyperparathyroidism and in some other diseases in which there were bone changes, also in acute rheumatic fever, in polyarteritis nodosa, in malabsorption syndrome, and in Marfan's syndrome. Free hydroxyproline was increased greatly as compared with total hydroxyproline in the urine of two patients with hyperparathyroidism and in two patients with azotemia. The determination

of total hydroxyproline in the urine and free hydroxyproline in the serum is of value in diagnosis in thyroid diseases and Marfan's syndrome, in following the response to therapy in thyroid diseases, acromegaly, and growth hormone treatment, and in the evaluation of the extent of pathology alterations in various diseases affecting the bones. All of the hydroxyproline determinations were performed by the method of Prockop and Udenfriend (61) with some practical modifications.

Table I shows the excretion of free and total urinary hydroxyproline and the values of serum free hydroxyproline in several disease conditions. Increased values were observed in hyperthyroidism, in acromegaly, in hyperparathyroidism, in Turner's syndrome, in Paget's disease of bone, in cancer with bone metastas, in myeloid leukaemia, in acute rheumatic fever, in polyarteritis nodosa, in Marfan's syndrome. Decreased values were found in hypothyroidism. The values were normal in Cushing's disease, in Addison's disease, in Klinefelter's syndrome, in diabetes mellitus, in osteoporosis, in lymphocytic leukemia, in systemic lupus erythematosus, in active scleroderma, in rheumatoid arthritis, in myocardial infarction, in duodenal ulcer, in varicose ulcer of the leg, in psoriasis vulgaris, in arteriosclerosis and in sarcoidosis.

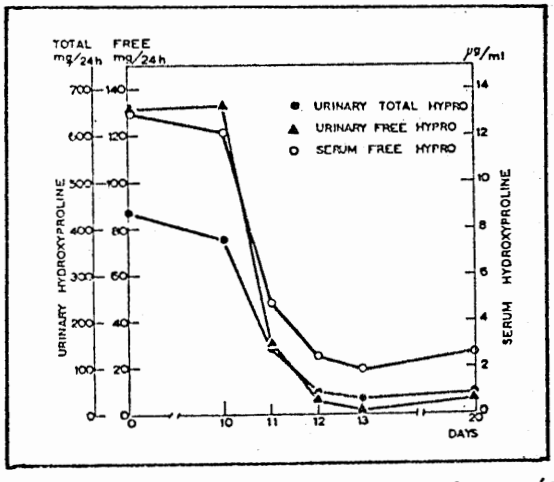
TABLE I

URINARY TOTAL AND FREE AND SERUM FREE HYDROXYPROLINE VALUES IN VARIOUS DISEASE CONDITIONS. URINARY TOTAL HYDROXYPROLINE IS EXPRESSED AS mg/24 hours/m^2, URINARY FREE HYDROXYPROLINE AS mg/24 hours AND SERUM FREE HYDROXYPROLINE AS µg/ml.

Condition	No	Total urinary HP	Free urinary HP	Serum free HP
Hyperthyroidism[1]	31	23.5—163.5		1.17— 3.99
Hypothyroidism[1]	5	5.2— 8.1		0.58— 0.96
Acromegaly	2	24.2, 44.7	0.88, 0.94	—, 1.17
Hyperparathyroidism	4	13.7, 17.3	0.83, 1.32	1.34, 1.37
		46.1, 218.0	7.20, 133.5	—, 12.10
Cushing's syndrome	1	10.6	1.35	0.84
Addison's disease	1	12.7	0.58	1.19
Turner's syndrome	1	55.4	1.17	1.54
Klinefelter's syndrome	1	19.2	0.63	—
Diabetes mellitus	2	9.2, 17.3	1.49, 1.13	0.91, 1.34
Paget's disease of bone	1	35.8	3.64	1.81
Osteoporosis	2	10.1, 25.0	0.37, 1.65	1.32, 1.80
Cancer with bone metastases	2	44.6, 46.6	0.82, 0.56	1.79, 2.10
Cancer with no bone metastases	1	20.1	0.82	0.87
Myeloid leukaemia	1	64.6	1.10	2.53
Systemic lupus erythematosus	3	11.3, 12.8	0.62, 0.47	0.97, 1.20
		19.0	0.65	—
Scleroderma	1	16.9	0.40	1.08
Rheumatoid arthritis	4	13.1, 13.5	0.37, 0.60	0.80, 0.78
		17.7, 23.9	0.74, 0.43	0.71, 1.33
Acute rheumatic fever	2	26.0, 33.0	0.74, 1.80	1.04, 1.55
Polyarteritis nodosa	1	29.4	1.45	1.43
Marfan's syndrome	2	19.9, 36.5	1.17, 0.88	1.50, 1.24
Malabsorption syndrome	3	40.0, 61.9	0.58, 1.03	2.04, 1.86
		96.0	1.32	2.94
Myocardial infarction	6	12.8, 12.8	0.58, 0.80	1.07, 1.58
		14.7, 15.4	0.76, 2.88	0.82, 0.92
		21.8, 26.9	0.40, 0.78	1.56, 0.93
Duodenal ulcer	1	14.0	1.19	0.97
Varicose ulcer of leg	1	15.2	0.95	1.50
Psoriasis vulgaris	1	19.4	1.37	1.47
Arteriosclerosis	3	10.1, 12.9	0.37, 0.64	1.32, 0.83
		19.0	0.69	1.18
Sarcoidosis	3	11.2, 12.0	0.89, 1.22	0.94, 1.41
		18.0	0.63	1.11
Azotaemia	2	11.1, 18.0	2.63, 3.56	1.84, 2.12

According to O. Laitinen and co-workers (50)

The hyperparathyroid patients with elevated hydroxyproline values had greatly altered proportions of free to total hydroxyproline in the urine. The free hydroxyproline consisted of 35.9 and 10.6 per cent of the total hydroxyproline. In one of these cases, which had the highest hydroxyproline values in the whole study, the urinary total and serum free hydroxyproline were increased about ten-fold, whereas urinary free hydroxyproline was increased over a 100-fold compared with the respective values in the normal subjects. In this patient a dramatic fall in all hydroxyproline values took place after operative removal of a parathyroid adenoma (Figure 4) (50).



According to Laitinen and co-workers (50)

FIGURE 4. EFFECT OF THE REMOVAL OF PARATHYROID ADENOMA ON THE HYDROXYPROLINE VALUES IN THE URINE AND SERUM. THE OPERATION WAS PERFORMED ON THE 10TH DAY AFTER TWO CONTROL DETERMINATIONS OF HYDROXYPROLINE.

In the two cases with severe renal azotaemia, high serum free and urinary free hydroxyproline values were observed in conjunction with a normal level of urinary total hydroxyproline excretion (50).

The results of the Laitinen study (50) indicate that hydroxyproline excretion is dependent on the age, sex and body size of the subject. The normal urinary total hydroxyproline which is excreted is well-known from previous studies (5) (22) (33) (66). None of these, however, were serum free and urinary free, with total hydroxyproline determined simultaneously. Ziff (73) has found that children excrete more hydroxyproline than adult subjects, but the influence of age on the hydroxyproline values in adult subjects has not been reported. The expression of the hydroxyproline excretion per unit of body surface area, suggested by Jasin (33), decreases the scatter of the urinary total hydroxyproline values and makes it possible to use the same normal values for both sexes. The free hydroxyproline in the serum has been less studied and normal values for serum free hydroxyproline have been presented only in one extensive study by Laitinen (50), in which a relatively complicated method of hydroxyproline determination was used. The results of the Laitinen study indicate that, besides the estimation of urinary total hydroxyproline, the determination of serum free hydroxyproline also is of clinical value. By contrast, the urinary free hydroxyproline seemed to give less information because of the great scatter of the normal values.

Recent studies with ^{14}C-proline indicate that the urinary free and total hydroxyproline are derived both from the recently synthesized collagen molecules, which form the so-called soluble collagen of the tissues, and from catabolism of the mature, insoluble collagen fibres (52) (59). The origin of the serum free hydroxyproline has not been studied with radioactive techniques; but a parallelism between the values of urinary and serum hydroxyproline suggests a similar origin (37) (60). This correlation also was noted in the study by Laitinen, particularly between the values for urinary total and serum free hydroxyproline (50).

The most remarkable deviations in the hydroxyproline values are encountered in endocrine disorders. This might be due to the fact that collagen synthesis, maturation, and degradation all are under hormonal control.

The urinary excretion of hydroxyproline has been found to be increased in acromegaly (5) (17) (33), and decreased in children with pituitary dwarfism (33). The administration of growth hormone to rats (41) and to pituitary dwarfs (33) has been reported to elevate, and x-ray therapy in acromegaly (33) to decrease the hydroxyproline values. On account of these findings, the measurement of hydroxyproline has been suggested to be a useful parameter of activity in acromegaly (68) and an index of response to growth hormone treatment (17). Because growth hormone is known to accelerate protein synthesis in general and to

increase the soluble collagen pool of the skin in hamsters (4), it seems reasonable that the elevated hydroxyproline values in acromegaly are due to increased formation and turnover of the recently synthesized collagen molecules.

Both normal and increased values of hydroxyproline in hyperparathyroidism have been observed in Laitinen's study (50), as well as in earlier reports (73) (43). High values have been found especially in patients with bone lesions (17). Increased excretion of total urinary hydroxyproline has been suggested to be due to increased bone collagen synthesis (36) (43), or to degradation of mature collagen secondary to mobilization of bone mineral (44). The rapid fall in the hydroxyproline values found after operation and the well-known increased bone formation and decreased lysis in hyperparathyroid patients after successful operations suggests that the hyperparathyroid effect is due mainly to degradation of mature collagen and not to increased collagen synthesis in osteitis fibrosa, as has been postulated (43).

Although cortisone has an inhibitory effect on the formation of collagen, the administration of cortisone to human subjects and adult rats (40) (48) has not been observed to influence hydroxyproline excretion. Neither have abnormal values been observed in Addison's or Cushing's disease. In young rats, however, cortisone greatly reduces the excretion of hydroxyproline in the urine, because of the reduced

proportion of urinary hydroxyproline derived from the newly synthesized collagen fractions (38) (39). Whether the low-normal values in some patients with Cushing's disease reported in this study and by Klein (48) could also be due to reduced synthesis of collagen cannot be stated, because of the small amount of relevant data reported for this disease.

In accordance with the findings of Benoit (5), both elevated and normal values were observed both in Turner's syndrome and in Klinefelter's syndrome, with high urinary pituitary gonadotropins. As suggested by Benoit, the elevated hydroxyproline values are more probably due to delayed maturity than to a direct effect of pituitary gonadotropins.

Increased urinary excretion of hydroxyproline has been observed in Laitinen's study (50) and other reports in several diseases affecting bone, such as Paget's disease (5) (17) (22), fibrous dysplasia of bone (22), cancer with bone metastases (48) (58), and rickets (45). The hydroxyproline values in osteoporosis may be normal or possibly slightly elevated (48) (51). The greatly increased value in one case of myeloid leukaemia in Laitinen's study (50) compared with the normal excretion in lymphatic leukaemia may be indicative of bone affection in the former disease. The above examples support the suggestion of Klein (43) and of Dull and Henneman (18) that changes in bone collagen alone can greatly alter the hydroxyproline excretion values. It seems apparent, however, that altered hydroxyproline values caused by some hormonal

actions, as by growth hormone, thyroid hormone, and cortisone are reflections of changes in the body collagen as a whole. In addition, there are a number of conditions, such as post-partum involution of the uterus (42) and extensive burns (47), in which large extraosseous changes in collagen cause increased values of hydroxyproline. By contrast, in small extraosseous connective tissue-forming processes, as in patients with myocardial infarction, duodenal ulcer and varicose ulcer of the leg, no change in the excretion of hydroxyproline can be found.

Patients with connective tissue disorders have shown normal or slightly elevated urinary hydroxyproline excretion (73). The results of Laitinen agree with these observations (50). Significantly increased values have been reported only in "active" scleroderma (63). Laitinen and several other workers (50) have not been able to confirm this finding (68). Nevertheless, the significantly elevated excretion in two cases of acute rheumatic fever and one case with polyarteritis nodosa in Laitinen's study (50) indicates that probably some of the collagen diseases might be accompanied by moderately altered values in the active phase of the disease.

Hydroxyproline excretion is elevated in the majority of the tested cases of Marfan's syndrome (34) (66). The determination of hydroxyproline might be of value if there are diagnostic difficulties in this disease.

The high hydroxyproline values in the malabsorption syndrome in the two young women might be due to the delayed maturity, secondary to malabsorption. Elevated values also were found in an older woman. Further, altered values in patients with tropical sprue have been reported (64). Therefore, an elevating mechanism primarily caused by these gastrointestinal disturbances cannot be excluded. It is possible that changes in the calcium metabolism of the patients with malabsorption give an explanation of the high value.

The normal hydroxyproline values found in patients with psoriasis vulgaris, arteriosclerosis and sarcoidosis indicate that, if present, the change in collagen metabolism in these diseases is too small or slow to alter the hydroxyproline values significantly.

In the two cases with azotaemia, urinary and serum free hydroxyproline were markedly increased as compared with the urinary total hydroxyproline values, which were within the normal range. Finlayson (20) reported lowered values of urinary total hydroxyproline excretion in five patients with acidotic uraemia. These findings could be interpreted as evidence of an increased degradation of peptide-bound hydroxyproline to free hydroxyproline in azotaemia.

The parallelism in the values of urinary and serum hydroxyproline suggests that the changes in these values are in general due to altered collagen metabolism and not to renal changes. In normal subjects

urinary free hydroxyproline like any free amino acid, is reabsorbed almost completely in the renal tubules. The greatly altered proportion of free to total hydroxyproline in the urine of two patients with hyperparathyroidism indicates, however, that there may be a defect in the reabsorption mechanism of free hydroxyproline in these cases. Excretion of peptide-bound hydroxyproline, which has been observed to be secreted by the renal tubules in loading tests (5), has not been proved to be altered on the renal level in any disease condition.

MENTAL RETARDATION

Large amounts of free hydroxyproline were found to accumulate in the blood and urine of a mentally retarded girl 13 years of age.

The urinary hydroxyproline was identified as L-4-hydroxyproline, the amino acid that constitutes 14 per cent of collagen. The patient had no apparent collagen disease, and the peptide-bound hydroxyproline, which is known to reflect collagen turnover, was normal.

Evidence that the accumulation of free hydroxyproline in the patient is the result of deficient activity of the enzyme hydroxyproline oxidase, which normally catalyzes the first step in hydroxyproline degradation, is presented.

The relation between the mental retardation and the elevated hydroxyproline concentration is not established. This is the only known

patient with the disorder, and the biochemical defect was discovered in the course of a survey of a retarded population.

The blood hydroxyproline concentration was not lowered by a hydroxyproline-free diet. No therapy is at present available for this disorder (19).

URINARY HYDROXYPROLINE: CREATININE RATIO

The urinary hydroxyproline excretion from normal individuals ranging from birth to 70 years of age was expressed as mg. of hydroxyproline per mg. of creatinine excreted per 24 hours. The hydroxyproline: creatinine ratio corrects, at least partially, for differences in body size between individuals, yielding a narrower range of values than the range of the urinary hydroxyproline values alone. A definite relation of the hydroxyproline:creatinine ratio with age was found, increasing from birth to one month of age, decreasing from six months to about five years of age, remaining constant to puberty, decreasing again to about age 20, and subsequently remaining constant through age 70 years. At all ages, the ratio is essentially equivalent in both sexes; and the variability of the ratio between individuals was much less than that of the corresponding 24-hour urinary hydroxyproline values. In the same individual, the variation of the ratio determined on multiple samples was also less than the variation observed in the two-hour urinary hydroxyproline from the same specimens (1).

CONCLUSIONS FROM THE LITERATURE

It seems reasonable to conclude that the level of excretion of urinary hydroxyproline peptides reflects the metabolism of collagen and that processes such as growth, which accelerate collagen formation with concomitant increase in the metabolically active extractable forms of collagen, are associated with moderate increases in peptide excretion. In addition, collagen-degrading processes may result in greater levels of excretion. In those conditions, such as hyperparathyroidism, Paget's disease, and even growth in which both synthesis and breakdown actively occur, it is difficult to make an evaluation of the relative contribution of the two processes to the urinary hydroxyproline. Excretion in the normal adult probably reflects the base-line contribution of mature collagen breakdown, but conditions increasing the soluble collagen fraction show an increase in urinary hydroxyproline excretion out of proportion to the actual mass of collagen this fraction represents. Whether this increment in excretion is a result of the high turnover rate of the soluble collagen pool alone or whether it also reflects an increase in turnover of the mature collagen fractions is unknown at present. In spite of these areas, which remain to be clarified, urinary hydroxyproline excretion appears to provide a valuable index of collagen metabolism in the intact animal.

Urinary hydroxyproline values have been observed to have a relatively constant level, with small variations in normal subjects and patients with several diseases. The characteristic changes in some diseases, however, suggest that the determination of urinary total hydroxyproline and serum free hydroxyproline may be of value in the following cases (51).

a. In diagnosis and treatment of thyroid diseases. As a diagnostic aid the method is especially useful in cases where the determination of proteinbound iodine is not possible. The effect of therapy is rapidly reflected in the hydroxyproline values.

b. As an index of response to therapy in growth hormone treatment and as an indicator of activity of acromegaly.

c. As a diagnostic aid in Marfan's syndrome.

d. When evaluating the extent of bone manifestations in various diseases.

PLAN OF PROCEDURE

PERIODS OF STUDY

Under the auspices of the National Aeronautics and Space Administration, the Texas Woman's University Research Institute has been conducting a series of bed rest studies. These studies are part of a vast research program being conducted in an effort to examine the response of subjects to conditions which simulate those which will be encountered in participation in the space flights.

This particular study lasted 97 days and included the following periods:

Equilibration Period, 29 days, June 19 - July 18, 1967.

Bed Rest Number One, 14 days, July 18 - August 1, 1967.

Interim Ambulatory Period, 14 days, August 1 - August 15, 1967.

Bed Rest Number Two, 14 days, August 15 - August 29, 1967.

Post-Bed Rest Period, 26 days, August 29 - September 23, 1967.

SUBJECTS OF THE STUDY

Chosen for this study were five male university students. Before the selection for participation in the study, these young men underwent extensive examinations, both physical and psychological. The following table shows their respective ages, heights and weights upon entering the study.

Subject	Age	Weight (lbs.)	Height (inches)
AA	24	155	70 1/4
BB	21	151	71 1/4
CC	21	138	66
DD	22	163	67
EE	21	182	73 1/2

DIET AND REGIMEN OF THE SUBJECTS

During the entire study, the subjects were housed and fed at the metabolic ward of the Nelda Childers Stark Laboratory for Human Nutrition Research at the Texas Woman's University Research Institute. Specially trained dietitians planned and supervised the preparation of the meals which were nutritionally adequate in all nutrients, calcium being the nutrient which was variable. A careful record was kept of the food intake of the individual subjects throughout the study.

This study was conducted under close medical supervision. Periodic x-rays were made as well as various clinical laboratory tests. A record was made of height and weight changes throughout the study. Specially trained orderlies attended to the hygienic needs of the subjects when immobilized.

Equilibration Period

During this span of 29 days, the subjects led a normal life. They were engaged in conducting various tasks in the laboratory eight hours daily. Their meals were planned to contain 800 mg. calcium/day during this period.

Bed Rest Number One

For a period of 14 days, the subjects were immobilized. They assumed a horizontal position on a single bed equipped with one pillow. They were encouraged not to lift their heads. Limited arm and leg movement was allowed. They were provided with hospital type television sets and given glasses equipped with prismatic lenses for reading. During this period the orderlies cared for the hygienic needs of the subjects. The young men were spoon fed, and a careful record was kept of their individual intake of food. Ca^{47} was incorporated into their milk intake the first morning of this period. Diets were planned to contain 800 mg. calcium.

Interim Ambulatory Period

During this period, the young men again were ambulatory. Four hours daily were spent in performing tasks assigned in the laboratory. Supervised physical activity was compulsory in the afternoons. Again, meals were served under the dietitian's supervision in the metabolic ward and were planned to contain 800 mg. calcium daily.

Bed Rest Number Two

The same conditions prevailed during this 14-day period as described under Bed Rest Number One, with the exception of the calcium content of the diet. In this phase of the study, the daily intake of calcium was lowered to 300 mg. As in the previous bed rest, Ca^{47} was incorporated into the milk the first morning of recumbency.

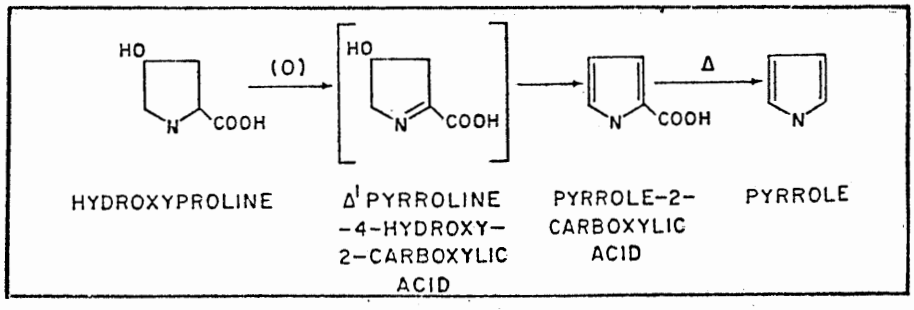
Post-Bed Rest Period

Conditions during this period were similar to those during the interim ambulatory period with regard to work and physical activity. During this time, the calcium intake was varied as follows: August 29-September 13 (1500 mg.), September 13-16 (300 mg.) (Bed Rest 2), September 16-23 (1500 mg.).

METHODOLOGY FOLLOWED IN HYDROXYPROLINE ANALYSIS

Since nearly all animal hydroxyproline is found in collagen, the measurement of hydroxyproline has been used extensively in studies on collagen formation and metabolism. A number of methods have been reported for the specific assay of the imino acid (57) (56) (69) (24) (31), but in general they can be used only with relatively pure solutions (61).

Most of the published methods for hydroxyproline involve the oxidation of the imino acid to pyrrole-2-carboxylic acid or pyrrole, and then formation of a chromophore with p-dimethylaminobenzaldehyde (Figure 5). Although the available methods based on this reaction are sensitive to 0.01 μmole hydroxyproline, results in urine and unpurified solutions are erratic largely because the oxidation step is difficult to control. Other substances which undergo oxidation destroy the oxidant and drastically reduce the yields of pyrrole (24); with an excess of



According to Prockop and Udenfriend (61)

FIGURE 5. OXIDATION AND DECARBOXYLATION OF HYDROXYPROLINE TO PYRROLE.

oxidant the desired product polymerizes (12). A further problem in many of the methods is that pigments and other products from biological materials which obscure the pyrrole chromophore (56).

In the procedure described here, hydroxyproline is oxidized in the presence of a measured excess of alanine, and as a result the varying amounts of amino acids or similar substances in urine or tissue hydrolyzates do not influence the yield of pyrrole. Specificity is achieved by extracting the pyrrole into toluene. Since the first oxidation products [Δ'-pyrroline-4-hydroxy-2-carboxylic acid pyrrole-2-carboxylic acid (62)] are not soluble in toluene, materials which might interfere with the color reaction are removed with toluene before the pyrrole is formed. These precautions make it possible to analyze 5 µg hydroxyproline in solutions containing over 50 mg. of other amino acids.

PROCEDURE ADOPTED IN THE TWU LABORATORIES

1. Urine Collection and Storage

A 24-hour urine specimen is collected in a plastic bottle. The name of the subject, the volume and date are recorded on the plastic bottle with the urine kept in the frozen state until ready for extraction. No chemical preservative is added to the samples during this study.

When the specimen is removed from the deep freeze, it is permitted to thaw. Then the specimen is thoroughly mixed before an aliquot is placed in a pyrex test tube for analysis.

2. Total Urinary Hydroxyproline:

   A. From 0.1 to 1.0 milliliters of urine is placed in a pyrex test tube, and each sample is analyzed in triplicate.

   B. Add 2.0 milliliters concentrated hydrochloric acid (hydrolysis is maximum at 6N).

   C. Seal tubes with a blow torch. These can be stored for several days in the deep freeze.

   D. Autoclave for 3 hours (124°C), store in the deep freeze (-20°C) until analyzed.

3. Removal of Pigments

   A. Break seal on autoclaved samples with pliers and towel.

   B. Pour contents of tubes into round bottom glass centrifuge tubes. (Note: if a small break has occurred in a tube during autoclaving the volume may be increased due to condensation of water during cooling. Make note of such a tube but run sample through entire procedure; however, rather than adding 4.0 milliliters distilled water, make up to 8.0 milliliters according to graduations of centrifuge. If the samples duplicate well, results may be interpreted as valid.)

C. Add 4.0 milliliters of distilled water to the total volume of 8.0 milliliters (final normality should not be less than 2 normal).

D. Add an amount not exceeding the equivalent of 1 milliliter Dowex-Charcoal resin (0.5 milliliter usually is sufficient) and mix well by repeated inversion using parafilm.

E. Centrifuge 15 minutes at 2,000 RPM. (During centrifugation standards and blanks may be made).

F. Take 4.0 ml. of supernatant liquid immediately with 4 ml. volumetric pipette, being careful not to disturb resin precipitate, and put into large culture tubes. These with standards and blank may be stored for one or two days at -20°C.

4. Preparation of Standard and Reagent Blanks

A. Stock standard is 100 µmole hydroxyproline per ml. Working standard is made at time of pigment removal by adding 0.1 ml. of 100 µmole/ml. stock standard to a 10 ml. volumetric flask and making to volume with distilled water. Working standard thus contains 1.0 µmole per ml. Range of the colorimetric determination is from 0.1 to 1.0 µmole. Usually two 0.5 µmole standards are used:

0.5 ml. working standard is placed in a centrifuge tube and 5.5 ml. of distilled water is added (to make to 6.0 ml.) followed by 2.0 ml. concentrated HCl. This is mixed and 4.0 ml. are added to a

large culture tube at the same time as decolorized samples are being put in culture tubes. Degeneration during storage therefore will reflect in the standard.

B. Reagent blank: 6.0 ml. distilled water plus 2.0 ml. HCl, mixed with 4.0 ml. added to a culture tube. Two blanks are sufficient.

5. Colorimetric Analysis

A. Allow samples in culture tubes to come to room temperature.

B. Add one drop 1% phenolphthalein.

C. Add 2.5 ml. 5N KOH.

D. Add 1N KOH dropwise to faint pink color. Colors may vary, but if only one drop has produced color, pH will not be sufficiently high to alter reactions, since buffer will be added later.

E. Add KCl in minimal excess to saturation (2.5 to 3.0 cc of granular salt).

F. Add 2 ml. borate buffer.

G. Add 1 ml. 10% alanine. Mix with the Vortex.

H. Add 2 ml. 0.2 M chloramine T, made fresh daily.

I. Let oxidation proceed at room temperature for 20 minutes.

J. After 20 minutes, reaction is stopped by adding 6.0 ml. sodium thiosulfate solution. Mix with Vortex.

K. Add approximately 10 ml. toluene and shake at high speed with shaker for 2 minutes. (At this point turn on boiling water bath.)

L. Centrifuge 10 minutes at 750 RPM.

M. Remove toluene phase via vacuum, being careful not to remove any of aqueous phase, even if this requires leaving a small residual of toluene.

N. Cap tubes and place in boiling water bath for 30 minutes.

O. Cool under running tap water. When next step is instituted, procedure must be carried straight through to completion.

P. Add exactly 10 ml. of toluene.

Q. Shake 2 minutes on shaker at high speed. (Check to make sure Coleman is on at this point.)

R. Centrifuge 10 minutes at 750 RPM.

S. Take exactly 5 ml. clear toluene phase with a 5 ml. volumetric pipette and put in Coleman test tube.

T. Add precisely 2.0 ml. Ehrlich's reagent using a 10 ml. serological pipette. Mix with Vortex.

U. Allow to stand 15 minutes.

V. Read at 560 mu on Coleman. (Valid range of OD readings is from 0.150 to 1.50 although best if below 1.0.)

6. Calculations

A. $K = \frac{\text{Grams of hydroxyproline in 25 } \mu\text{m/ml solution of standard}}{\text{OD Standard}}$

B. $K \times OD = \frac{\text{milligrams of hydroxyproline}}{100 \text{ milliliters}}$

C. $\frac{\text{milligrams of hydroxyproline}}{100 \text{ milliliters}} \times \frac{\text{total volume of urine}}{100} =$

mg. of hydroxyproline/24 hrs.

COMMENTS:

1. Accuracy is required when preparing samples for autoclaving and at each step through addition of 4 ml. decolorized sample to culture tubes. Thereafter precision is not as critical, since the final product is extracted into a constant volume of 10 ml. of toluene. Then toluene and final color reaction reagents again must be added carefully,

2. Storage at -20° at various stages as described is not known to be harmful to stability of hydroxyproline; but length of time these remain stable is not worked out. The less storage time, in general, the better. Where there are known limitations these have been noted in the procedure above.

3. If too much KCl is added there often is difficulty in separating aqueous and toluene phases, and also, any KCl present when color reagent is added will cause failure. Care also must be taken not to use too little.

4. Reagents used in massive amounts so that close watch should be kept on available stock are: sodium thiosulfate, toluene, d- alanine, p-dimethylaminobenzaldehyde, and KOH.

5. It is recommended that the paper of Prockop and Udenfriend from Analytical Biochemistry 1:228-239 (1960) entitled Analysis of Hydroxyproline be read before procedure is attempted so that theory presented there and further application of the procedure can be appreciated.

6. Amounts of Ehrlich's reagent and toluene phase have been reduced in this procedure from those described in the reported method to (a) conserve Ehrlich's reagent, (b) produce a handy working volume, and (c) allow for enough toluene phase remaining to repeat this step if necessary. Do not discard contents of culture tubes until reading has occurred for reason (c) above.

REAGENTS

(1) Borate buffer, pH 8.7 -- 61.84 g. boric acid and 225 g. potassium chloride are mixed into about 800 ml. of distilled water. The pH is adjusted to 8.7 with a 5 normal potassium hydroxide solution and the final volume is made up to 1 liter.

(2) Alanine Solution -- Ten grams of alanine are dissolved in about 90 ml. of distilled water, the pH is adjusted to 8.7 with a 5 normal

potassium hydroxide solution, and the final volume is made up to 100 ml.

(3) Chloramine T Solutions -- (Eastman Organic Chemicals) 0.2 M solution in Methyl Cellosolve is prepared daily. 2.8 g. chloramine T made to 50 ml. with methyl cellosolve, then mix thoroughly.

(4) Sodium Thiosulfate -- Solution is 3.6 M in distilled water and is stored under toluene at room temperature for several weeks.

(5) p-Dimethylaminobenzaldehyde or Ehrlich's Reagent -- (Analytical grade; Matheson, Coleman & Bell). Concentrated sulfuric acid, 27.4 ml., is slowly added to 200 ml. of absolute alcohol in a beaker and the mixture cooled. p-Dimethylaminobenzaldehyde, 120 g., is added to 200 ml. of absolute alcohol in another beaker, and then the acid-ethanol mixture is slowly stirred into the first beaker. The solution can be stored in the refrigerator for several weeks; the crystals which precipitate on cooling are redissolved by warming the solution briefly.

(6) Dowex-Charcoal Resin -- Forty g. analytical grade Dowex 1 and 20 g. Norit A added to about 100 ml. 6 N HCl. Wash several times with 6N HCl in Buchner funnel until effluent is no longer yellowish. Dry with several rinses of 1:1 ethanol:ether.

(7) Hydroxy-L-Proline -- 1.31 g. of hydroxyproline is mixed into about 90 milliliters of distilled water. The final volume is made up to 100 ml. and stored in the refrigerator.

(8) Phenolphthalein -- One gram of phenolphthalein is mixed into about 50 ml. of ethanol. The final volume is made up to 100 ml.

(9) Potassium Chloride -- Analytical grade, granular.

(10) Toluene -- Reagent grade.

(11) Potassium Hydroxide -- (5 normal) 280.55 g. of potassium hydroxide is dissolved in about 900 ml. of distilled water. The final volume is made up to 1 liter.

(1 normal) 56.11 grams of potassium hydroxide is dissolved in about 900 milliliters of distilled water. The final volume is made up to 1 liter.

PRESENTATION OF DATA WITH DISCUSSION

Table II gives the data concerning urinary excretion of hydroxyproline for Bed Rest 1 and the related ambulatory periods. Table III gives the comparable data for Bed Rest 2. Table III summarizes the statistical findings when the different periods of the study were compared for the respective subjects and for pooled data from all of the subjects.

COMPARISON OF EXCRETION OF HYDROXYPROLINE DURING BED REST 1 WITH THE PRE-BED REST AMBULATORY EQUILIBRATION PERIOD

The data showed that more hydroxyproline was excreted in the urine during Bed Rest 1 for each subject than during the immediate Pre-Bed Rest Period. The difference was statistically significant for Subjects AA, CC, and DD. With the data of all five subjects pooled together, the difference was highly significant in behalf of the bed rest period being greater, by a highly significant difference ($P < 0.001$).

COMPARISON OF FINDINGS FROM BED REST 2 AND THE INTERIM AMBULATORY PERIOD

The quantity of hydroxyproline excreted in the urine was greater during Bed Rest 2 than during the Interim Equilibration Period which immediately preceded the bed rest for all subjects, with differences which

were statistically significant in all cases except one. When the data for the five subjects were pooled, the urinary excretion of hydroxyproline during Bed Rest 2 surpassed that of the Interim Ambulatory Period by a difference which was highly significant ($P < 0.001$).

COMPARISON OF URINARY EXCRETION OF HYDROXYPROLINE DURING THE TWO BED REST PERIODS

The amounts of hydroxyproline excreted in the urine during Bed Rest 2 when only 300 mg. of calcium were fed were compared with that excreted during Bed Rest 1 when 800 mg. were provided, there was no statistically significant difference between the two levels when data from all subjects were pooled. Nor was there any statistically significant difference between the two bed rest periods for four of the individual subjects. In the case of Subject AA, the amount of urinary hydroxyproline excreted during Bed Rest 2 surpassed that of Bed Rest 1 ($P < 0.05$).

COMPARISON OF AMBULATORY PERIODS

There were only minor differences between the quantity of hydroxyproline excreted during the various ambulatory periods, with the differences not always in the same direction.

COMPARISON OF BED REST PERIODS WITH THE FINAL AMBULATORY RECONDITIONING PERIOD

Both the first and second bed rest periods surpassed the final ambulatory period in urinary hydroxyproline excretion for all subjects. The difference was not statistically significant for two subjects, but with the data for all five subjects pooled, the excretion of hydroxyproline in the urine was higher for Bed Rest 1 and for Bed Rest 2 than for the final ambulatory period by a highly significant difference ($P < 0.001$ in both comparisons).

SUMMARY AND CONCLUSIONS

A study designed to test various physiologic effects of immobilization on five healthy males was carried out. This study was composed of two Bed Rest Periods, using ^{47}Ca as a tracer. In the first period an 800 milligram calcium diet/day was used, while Bed Rest 2 was characterized by a 300-milligram calcium diet/day, to test the performance during bed rest on changes in bone density, calcium-phosphorus metabolism and urinary excretion of nitrogen, creatine, creatinine, 17-ketosteroids, 17-hydroxycorticosteroids, and hydroxyproline. A Pre-Bed Rest Period, an Interim Period, and a Post-Bed Rest Period during ambulation were used for comparison with the two Bed Rest Periods; with the two Bed Rest Periods compared with each other. The work of the author was the measurement of urinary hydroxyproline throughout the study.

During the ambulatory periods, the mean value tended to fall far below the mean value during both bed rest periods.

Urinary hydroxyproline values were increased in all subjects during the first Bed Rest. All subjects had higher hydroxyproline during the first Bed Rest than during the Pre-Bed Rest Period, with three subjects, Subject AA, Subject CC, and Subject DD experiencing increases which were statistically significant. When the excretion values for all subjects were pooled together for each period, it was shown that the

excretion during Bed Rest.1 was higher than during the Pre-Bed Rest Period by a difference which was statistically highly significant.

A decrease in urinary hydroxyproline was noted during the Interim Equilibration Ambulatory Period in all subjects. Subject CC showed that the average excretion during this period was lower than in any other period in this study. The overall average for this period was 53.46 milligrams/24 hours as compared to 53.76 milligrams/24 hours during the Pre-Bed Rest Period, 63.25 milligrams/24 hours during Bed Rest 1, 68.69 milligrams/24 hours during Bed Rest 2, and 51.53 milligrams/24 hours during the Post-Bed Rest Ambulatory Period. The decrease during this period was statistically significant for all subjects when compared with Bed Rest 1. During Bed Rest 2 the overall value for all subjects was higher than during the previous ambulatory period by a difference which was highly significant.

The second Bed Rest Period during which the 300 milligram calcium diet/day was used resulted in a slight increase in urinary hydroxyproline in all five subjects. This increase was statistically highly significant when compared with the Pre-Bed Rest Period, the Interim Ambulatory Period, and the Post-Bed Rest Ambulatory Period.

All subjects showed a decrease in urinary hydroxyproline excretion during the Post-Bed Rest Ambulatory Period, with this decrease

greater than that experienced during the Interim Period. This decrease was statistically highly significant when compared with the Bed Rest 1 and Bed Rest 2 Periods.

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A P P E N D I X

TABLE II

URINARY HYDROXYPROLINE EXCRETION WHEN 800 MILLIGRAMS OF CALCIUM WERE PROVIDED

PART A. SUBJECT AA

PRE-BED REST		14-DAY BED REST	
Date	Excretion of Urinary Hydroxyproline (mg. per 24 hours)	Date	Excretion of Urinary Hydroxyproline (mg. per 24 hours)
June 19	73.22	July 18	35.21
20	52.48	19	34.74
21	53.26	20	56.12
22	32.92	21	72.44
23	21.11	22	55.76
24	48.08	23	74.75
25	58.03	24	37.40
26	52.80	25	46.98
27	51.82	26	102.53
28	47.15	27	33.89
29	50.05	28	96.26
30	47.50	29	69.83
July 1	43.50	30	31.40
2	53.02	31	109.90
3	38.76	Mean	61.23
4	29.20		
5	34.50	POST-BED REST	
6	44.46		
7	49.92	August 1	67.52
8	54.50	2	69.76
9	42.47	3	68.08
10	46.98	4	43.30
11	35.39	5	60.75
12	49.75	6	36.30
13	48.20	7	42.59
14	48.61	8	52.18
15	48.64	9	57.47
16	63.22	10	73.53
17	54.26	11	77.72
		12	55.70
		13	50.97
		14	48.61
Mean	47.37	Mean	57.46

TABLE II, CONTINUED

URINARY HYDROXYPROLINE EXCRETION WHEN 800 MILLIGRAMS OF CALCIUM WERE PROVIDED

PART B. SUBJECT BB

PRE-BED REST		14-DAY BED REST	
Date	Excretion of Urinary Hydroxyproline (mg. per 24 hours)	Date	Excretion of Urinary Hydroxyproline (mg. per 24 hours)
June 19	70.04	July 18	45.76
20	66.82	19	28.57
21	64.45	20	85.02
22	65.22	21	84.67
23	70.98	22	72.49
24	85.41	23	49.35
25	61.23	24	90.71
26	69.94	25	56.66
27	77.85	26	80.04
28	52.89	27	51.87
29	56.05	28	87.47
30	53.73	29	69.51
July 1	63.77	30	46.40
2	47.49	31	64.06
3	42.46	Mean	65.18
4	46.45		
5	44.59	POST-BED REST	
6	41.92		
7	53.82	August 1	45.35
8	51.74	2	68.88
9	49.59	3	64.02
10	44.01	4	49.54
11	49.73	5	52.01
12	55.20	6	68.65
13	39.28	7	63.89
14	49.45	8	54.38
15	39.80	9	49.62
16	81.38	10	51.73
17	64.30	11	57.63
		12	41.39
		13	46.93
Mean	57.23	14	49.45
		Mean	54.53

TABLE II, CONTINUED

URINARY HYDROXYPROLINE EXCRETION WHEN 800 MILLIGRAMS OF CALCIUM WERE PROVIDED

PART C. SUBJECT CC

PRE-BED REST		14-DAY BED REST	
Date	Excretion of Urinary Hydroxyproline (mg. per 24 hours)	Date	Excretion of Urinary Hydroxyproline (mg. per 24 hours)
June 19	62.08	July 18	52.94
20	54.68	19	56.46
21	54.67	20	64.51
22	58.82	21	62.47
23	55.54	22	60.21
24	57.55	23	37.48
25	61.72	24	70.88
26	62.55	25	63.54
27	60.64	26	81.38
28	53.20	27	37.21
29	53.36	28	73.46
30	48.86	29	52.77
July 1	45.07	30	51.34
2	45.80	31	62.27
3	43.14	Mean	59.07
4	48.02		
5	46.54	POST-BED REST	
6	49.32		
7	44.15	August 1	46.13
8	41.27	2	46.44
9	43.23	3	62.70
10	48.76	4	21.02
11	39.14	5	45.27
12	31.37	6	26.16
13	40.33	7	24.94
14	42.42	8	22.52
15	44.36	9	21.30
16	47.50	10	26.15
17	47.62	11	21.73
		12	24.21
		13	23.90
Mean	49.37	14	22.42
		Mean	31.06

TABLE II, CONTINUED

URINARY HYDROXYPROLINE EXCRETION WHEN 800 MILLIGRAMS OF CALCIUM WERE PROVIDED

PART D. SUBJECT DD

PRE-BED REST		14-DAY BED REST	
Date	Excretion of Urinary Hydroxyproline (mg. per 24 hours)	Date	Excretion of Urinary Hydroxyproline (mg. per 24 hours)
June 19	57.04	July 18	45.24
20	43.63	19	25.86
21	56.83	20	75.15
22	67.54	21	65.91
23	73.14	22	68.66
24	80.01	23	33.53
25	53.66	24	91.66
26	61.45	25	43.39
27	70.75	26	64.09
28	57.69	27	58.31
29	54.18	28	90.88
30	54.56	29	55.62
July 1	54.90	30	55.94
2	45.00	31	59.80
3	32.77	Mean	59.57
4	33.53		
5	47.40	POST-BED REST	
6	47.68		
7	46.48	August 1	60.62
8	52.01	2	59.83
9	39.14	3	59.11
10	41.97	4	53.75
11	36.71	5	43.32
12	36.58	6	55.50
13	39.85	7	56.72
14	48.56	8	59.16
15	51.30	9	60.38
16	57.82	10	55.21
17	47.12	11	52.67
		12	43.41
		13	42.22
		14	48.56
Mean	51.36	Mean	54.32

TABLE II, CONTINUED

URINARY HYDROXYPROLINE EXCRETION WHEN 800 MILLIGRAMS OF CALCIUM WERE PROVIDED

PART E. SUBJECT EE

PRE-BED REST		14-DAY BED REST	
Date	Excretion of Urinary Hydroxyproline (mg. per 24 hours)	Date	Excretion of Urinary Hydroxyproline (mg. per 24 hours)
June 19	78.34	July 18	59.29
20	73.32	19	54.58
21	76.98	20	81.43
22	79.34	21	86.31
23	82.35	22	83.16
24	79.06	23	56.89
25	66.37	24	52.16
26	65.19	25	66.75
27	63.85	26	69.83
28	55.13	27	52.58
29	57.04	28	111.99
30	55.29	29	79.94
July 1	60.27	30	95.04
2	60.00	31	47.08
3	61.01	Mean	71.22
4	47.03		
5	42.56	POST-BED REST	
6	35.95		
7	50.76	August 1	56.83
8	62.10	2	69.83
9	61.53	3	98.31
10	54.22	4	79.03
11	43.75	5	65.51
12	43.55	6	66.24
13	41.08	7	71.54
14	89.54	8	82.13
15	82.08	9	87.43
16	87.79	10	77.42
17	85.28	11	66.31
		12	53.72
		13	54.71
Mean	63.47	14	59.54
		Mean	70.61

TABLE III

URINARY HYDROXYPROLINE EXCRETION WHEN 300 MILLIGRAMS OF CALCIUM WERE PROVIDED DURING THE BED REST WITH 800 MILLIGRAMS PROVIDED DURING THE PRE-BED REST AND 1500 MILLIGRAMS DURING THE POST-BED REST PERIODS

PART A. SUBJECT AA

PRE-BED REST		BED REST II	
Date	Excretion of Urinary Hydroxyproline (mg. per 24 hours)	Date	Excretion of Urinary Hydroxyproline (mg. per 24 hours)
August 1	67.52	August 15	46.23
		16	52.12
2	69.76	17	56.94
		18	106.53
3	68.08	19	110.58
		20	114.45
4	43.30	21	103.85
		22	87.56
5	60.75	23	84.11
		24	77.16
6	36.30	25	50.15
		26	126.14
7	42.59	27	77.36
		28	86.12
8	52.18	Mean	84.23
9	57.47	POST-BED REST II	
		August 29	79.05
10	73.53	30	68.12
		31	65.84
11	77.72	September 1	67.80
		2	61.47
12	55.70	3	57.29
		4	48.74
13	50.97	5	44.19
		6	52.33
14	48.61	7	54.98
		8	49.37
		9	45.95
		10	48.03
Mean	57.46	11	50.10
		12	56.66
		Mean	56.66

TABLE III, CONTINUED

URINARY HYDROXYPROLINE EXCRETION WHEN 300 MILLIGRAMS OF CALCIUM WERE PROVIDED DURING THE BED REST WITH 800 MILLIGRAMS PROVIDED DURING THE PRE-BED REST AND 1500 MILLIGRAMS DURING THE POST-BED REST PERIODS

PART B. SUBJECT BB

PRE-BED REST		BED REST II	
Date	Excretion of Urinary Hydroxyproline (mg. per 24 hours)	Date	Excretion of Urinary Hydroxyproline (mg. per 24 hours)
August 1	45.35	August 15	30.32
		16	34.90
2	68.88	17	37.06
		18	39.22
3	64.02	19	84.51
		20	34.84
4	49.54	21	52.08
		22	67.45
5	52.01	23	64.37
		24	88.66
6	68.65	25	66.46
		26	68.16
7	63.89	27	62.01
		28	75.27
8	54.38	Mean	57.52
		POST-BED REST II	
9	49.62	August 29	54.23
		30	57.09
10	51.73	31	51.98
		September 1	45.96
11	57.63	2	49.04
		3	53.78
12	41.39	4	56.55
		5	40.09
13	46.93	6	37.38
		7	38.75
14	49.45	8	45.96
		9	47.18
		10	49.03
Mean	54.53	11	50.88
		12	48.42
		Mean	48.42

TABLE III, CONTINUED

URINARY HYDROXYPROLINE EXCRETION WHEN 300 MILLIGRAMS OF CALCIUM WERE PROVIDED DURING THE BED REST WITH 800 MILLIGRAMS PROVIDED DURING THE PRE-BED REST AND 1500 MILLIGRAMS DURING THE POST-BED REST PERIODS

PART C. SUBJECT CC

PRE-BED REST		BED REST II	
Date	Excretion of Urinary Hydroxyproline (mg. per 24 hours)	Date	Excretion of Urinary Hydroxyproline (mg. per 24 hours)
August 1	46.13	August 15	23.59
		16	23.94
2	46.44	17	88.88
		18	47.60
3	62.70	19	58.56
		20	53.64
4	21.02	21	57.65
		22	56.47
5	45.27	23	70.94
		24	53.54
6	26.16	25	49.16
		26	63.07
7	24.94	27	57.80
		28	50.80
8	22.52	Mean	53.97
		POST-BED REST II	
9	21.30	August 29	52.47
		30	56.93
10	26.15	31	46.79
		September 1	32.62
11	21.73	2	27.32
		3	26.39
12	24.21	4	33.63
		5	31.79
13	23.90	6	41.92
		7	43.90
14	22.42	8	24.98
		9	23.58
		10	25.52
Mean	31.06	11	26.84
		12	35.33
		Mean	35.33

TABLE III, CONTINUED

URINARY HYDROXYPROLINE EXCRETION WHEN 300 MILLIGRAMS OF CALCIUM WERE PROVIDED DURING THE BED REST WITH 800 MILLIGRAMS PROVIDED DURING THE PRE-BED REST AND 1500 MILLIGRAMS DURING THE POST-BED REST PERIODS

PART D. SUBJECT DD

PRE-BED REST		BED REST II	
Date	Excretion of Urinary Hydroxyproline (mg. per 24 hours)	Date	Excretion of Urinary Hydroxyproline (mg. per 24 hours)
August 1	60.62	August 15	41.03
		16	47.07
2	59.83	17	61.72
		18	80.29
3	59.11	19	61.15
		20	63.19
4	53.75	21	65.22
		22	62.25
5	43.32	23	79.77
		24	75.74
6	55.50	25	55.35
		26	75.15
7	56.72	27	78.54
		28	60.13
8	59.16	Mean	64.76
		POST-BED REST II	
9	60.38	August 29	64.95
		30	65.70
10	55.21	31	65.61
		September 1	59.30
11	52.67	2	54.81
		3	43.75
12	43.41	4	42.79
		5	37.17
13	42.22	6	45.68
		7	54.23
14	48.56	8	44.73
		9	42.86
		10	43.16
Mean	53.60	11	43.45
		12	50.58
		Mean	50.58

TABLE III, CONTINUED

URINARY HYDROXYPROLINE EXCRETION WHEN 300 MILLIGRAMS OF CALCIUM WERE PROVIDED DURING THE BED REST WITH 800 MILLIGRAMS PROVIDED DURING THE PRE-BED REST AND 1500 MILLIGRAMS DURING THE POST-BED REST PERIODS

PART E. SUBJECT EE

PRE-BED REST		BED REST II	
Date	Excretion of Urinary Hydroxyproline (mg. per 24 hours)	Date	Excretion of Urinary Hydroxyproline (mg. per 24 hours)
August 1	56.83	August 15	53.81
		16	49.38
2	69.83	17	74.78
		18	87.32
3	98.31	19	78.64
		20	85.43
4	79.03	21	98.12
		22	77.63
5	65.57	23	113.18
		24	90.63
6	66.24	25	68.33
		26	98.79
7	71.54	27	98.89
		28	86.86
8	82.13	Mean	82.98
		POST-BED REST II	
9	87.43	August 29	74.43
		30	75.31
10	77.42	31	82.69
		September 1	77.53
11	66.31	2	64.60
		3	68.39
12	53.72	4	67.59
		5	69.29
13	54.71	6	76.83
		7	76.27
14	59.54	8	66.96
		9	57.23
		10	56.26
Mean	70.61	11	55.30
		12	69.19
		Mean	69.19

TABLE IV

STATISTICAL COMPARISON OF URINARY HYDROXYPROLINE BETWEEN PAIRS OF THE DIFFERENT PERIODS OF THE STUDY

PART A. SUBJECT AA

Populations Compared	Means	Standard Deviation	"t" Value	Probability
Pre-Bed Rest Period Bed Rest No. 1	47.37 61.23*	10.10 26.09	2.3621	$P < 0.02$
Pre-Bed Rest Period Interim Ambulatory	47.37 57.46*	10.10 12.15	2.7305	$P < 0.01$
Pre-Bed Rest Period Bed Rest No. 2	47.37 84.24*	10.10 24.98	6.4941	$P < 0.001$
Pre-Bed Rest Period Post-Bed Rest Ambulatory	47.37 54.12*	10.10 8.66	2.5170	$P < 0.02$
Bed Rest No. 1 Interim Ambulatory	61.23* 57.46	26.09 12.15	0.4548	N.S.
Bed Rest No. 1 Bed Rest No. 2	61.23 84.24*	26.09 24.98	2.2129	$P < 0.05$
Bed Rest No. 1 Post-Bed Rest Ambulatory	61.23* 54.12	26.09 8.66	1.1725	N.S.
Interim Ambulatory Bed Rest No. 2	57.46 84.24*	12.15 24.98	3.3483	$P < 0.01$
Interim Ambulatory Post-Bed Rest Ambulatory	57.46* 54.12	12.15 8.66	0.9429	N.S.
Bed Rest No. 2 Post-Bed Rest Ambulatory	84.24* 54.12	24.98 8.66	5.1481	$P < 0.001$

*Period which is greater

TABLE IV, CONTINUED

STATISTICAL COMPARISON OF URINARY HYDROXYPROLINE BETWEEN PAIRS OF THE DIFFERENT PERIODS OF THE STUDY

PART B. SUBJECT BB

Populations Compared	Means	Standard Deviation	"t" Value	Probability
Pre-Bed Rest Period Bed Rest No. 1	57.23 65.18*	12.47 18.49	1.5792	N.S.
Pre-Bed Rest Period Interim Ambulatory	57.23* 54.53	12.47 8.44	0.6992	N.S.
Pre-Bed Rest Period Bed Rest No. 2	57.23 57.52*	12.47 18.75	0.0581	N.S.
Pre-Bed Rest Period Post-Bed Rest Ambulatory	57.23* 47.63	12.47 5.85	3.3405	$P < 0.001$
Bed Rest No. 1 Interim Ambulatory	65.18* 54.53	18.49 8.44	1.8207	$P < 0.10$
Bed Rest No. 1 Bed Rest No. 2	65.18* 57.52	18.49 18.75	1.0110	N.S.
Bed Rest No. 1 Post-Bed Rest Ambulatory	65.18* 47.63	18.49 5.85	4.0396	$P < 0.001$
Interim Ambulatory Bed Rest No. 2	54.53 57.52*	8.44 18.75	0.5051	N.S.
Interim Ambulatory Post-Bed Rest Ambulatory	54.53* 47.63	8.44 5.85	2.8043	$P < 0.01$
Bed Rest No. 2 Post-Bed Rest Ambulatory	57.52* 47.63	18.75 5.85	2.2497	$P < 0.05$

*Period which is greater

TABLE IV, CONTINUED

STATISTICAL COMPARISON OF URINARY HYDROXYPROLINE BETWEEN PAIRS OF THE DIFFERENT PERIODS OF THE STUDY

PART C. SUBJECT CC

Populations Compared	Means	Standard Deviation	"t" Value	Probability
Pre-Bed Rest Period Bed Rest No. 1	49.37 59.07*	7.65 11.97	3.0483	$P < 0.01$
Pre-Bed Rest Period Interim Ambulatory	49.37* 31.06	7.65 12.76	5.5480	$P < 0.001$
Pre-Bed Rest Period Bed Rest No. 2	49.37 53.97*	7.65 15.91	1.2135	N.S.
Pre-Bed Rest Period Post-Bed Rest Ambulatory	49.37* 39.29	7.65 12.21	3.5467	$P < 0.001$
Bed Rest No. 1 Interim Ambulatory	59.07* 31.06	11.97 12.76	5.5605	$P < 0.001$
Bed Rest No. 1 Bed Rest No. 2	59.07* 53.97	11.97 15.91	0.8886	N.S.
Bed Rest No. 1 Post-Bed Rest Ambulatory	59.07* 39.29	11.97 12.21	4.6369	$P < 0.001$
Interim Ambulatory Bed Rest No. 2	31.06 53.97*	12.76 15.91	3.9024	$P < 0.001$
Interim Ambulatory Post-Bed Rest Ambulatory	31.06 39.29*	12.76 12.21	1.8831	$P < 0.10$
Bed Rest No. 2 Post-Bed Rest Ambulatory	53.97* 39.29	15.91 12.21	3.0500	$P < 0.01$

*Period which is greater

TABLE IV, CONTINUED

STATISTICAL COMPARISON OF URINARY HYDROXYPROLINE BETWEEN PAIRS OF THE DIFFERENT PERIODS OF THE STUDY

PART D. SUBJECT DD

Populations Compared	Means	Standard Deviation	"t" Value	Probability
Pre-Bed Rest Period Bed Rest No. 1	51.36 59.57*	11.54 18.34	1.6980	$P < 0.10$
Pre-Bed Rest Period Interim Ambulatory	51.36 53.60*	11.54 6.42	0.6511	N.S.
Pre-Bed Rest Period Bed Rest No. 2	51.36 64.76*	11.54 11.66	3.3909	$P < 0.001$
Pre-Bed Rest Period Post-Bed Rest Ambulatory	51.36* 45.16	11.54 9.46	2.0572	$P < 0.05$
Bed Rest No. 1 Interim Ambulatory	59.57* 53.60	18.34 6.42	1.0675	N.S.
Bed Rest No. 1 Bed Rest No. 2	59.57 64.76*	18.34 11.66	0.8288	N.S.
Bed Rest No. 1 Post-Bed Rest Ambulary	59.57* 45.16	18.34 9.46	3.0528	$P < 0.01$
Interim Ambulatory Bed Rest No. 2	53.60 64.76*	6.42 11.66	2.9122	$P < 0.01$
Interim Ambulatory Post-Bed Rest Ambulatory	53.60* 45.16	6.42 9.46	2.8317	$P < 0.01$
Bed Rest No. 2 Post-Bed Rest Ambulatory	64.76* 45.16	11.66 9.46	5.3958	$P < 0.001$

*Period which is greater

TABLE IV, CONTINUED

STATISTICAL COMPARISON OF URINARY HYDROXYPROLINE BETWEEN PAIRS OF THE DIFFERENT PERIODS OF THE STUDY

PART E. SUBJECT EE

Populations Compared	Means	Standard Deviation	"t" Value	Probability
Pre-Bed Rest Period Bed Rest No. 1	63.47 71.22*	15.16 18.36	1.3912	N.S.
Pre-Bed Rest Period Interim Ambulatory	63.47 70.62*	15.16 12.58	1.4575	N.S.
Pre-Bed Rest Period Bed Rest No. 2	63.47 82.99*	15.16 17.01	3.6161	$P < 0.001$
Pre Bed Rest Period Post-Bed Rest Ambulatory	63.47 66.42*	15.16 7.53	0.8489	N.S.
Bed Rest No. 1 Interim Ambulatory	71.22* 70.62	18.36 12.58	0.0939	N.S.
Bed Rest No. 1 Bed Rest No. 2	71.22 82.99*	18.36 17.01	1.6335	N.S.
Bed Rest No. 1 Post-Bed Rest Ambulatory	71.22* 66.42	18.36 7.53	1.0801	N.S.
Interim Ambulatory Bed Rest No. 2	70.62 82.99*	12.58 17.01	2.0313	$P < 0.10$
Interim Ambulatory Post-Bed Rest Ambulatory	70.62* 66.42	12.58 7.53	1.2312	N.S.
Bed Rest No. 2 Post-Bed Rest Ambulatory	82.99* 66.42	17.01 7.53	3.9509	$P < 0.001$

*Period which is greater

TABLE IV, CONTINUED

STATISTICAL COMPARISON OF URINARY HYDROXYPROLINE BETWEEN PAIRS OF THE DIFFERENT PERIODS OF THE STUDY

PART F. ALL SUBJECTS

Populations Compared	Means	Standard Deviation	"t" Value	Probability
Pre-Bed Rest Period Bed Rest No. 1	53.8 63.3*	13.0 19.7	4.1577	$P < 0.001$
Pre-Bed Rest Period Interim Ambulatory	53.8* 53.5	13.0 16.7	0.1442	N.S.
Pre-Bed Rest Period Bed Rest No. 2	53.8 68.7*	13.0 22.2	6.1267	$P < 0.001$
Pre-Bed Rest Period Post-Bed Rest Ambulatory	53.8* 51.5	13.0 14.2	1.1574	N.S.
Bed Rest No. 1 Interim Ambulatory	63.3* 53.5	19.7 16.7	3.1286	$P < 0.01$
Bed Rest No. 1 Bed Rest No. 2	63.3 68.7*	19.7 22.2	1.5129	N.S.
Bed Rest No. 1 Post-Bed Rest Ambulatory	63.3* 51.5	19.7 14.2	4.0756	$P < 0.001$
Interim Ambulatory Bed Rest No. 2	53.5 68.7*	16.7 22.2	4.5285	$P < 0.001$
Interim Ambulatory Post-Bed Rest Ambulatory	53.5* 51.5	16.7 14.2	0.7414	N.S.
Bed Rest No. 2 Post-Bed Rest Ambulatory	68.7* 51.5	22.2 14.2	5.5141	$P < 0.001$

*Period which is greater