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CLINICO-BIOCHEMICAL STUDY ON SENILE OSTEOPOROSIS

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Osteoporosis, except that occurring secondarily to known pathogenesis, is termed senile or postmenopausal osteoporosis and arises from a still unknown cause. Primary osteoporosis may occur as a result of adrenal-gonadal imbalance or of calcium deficiency. However since these pathogenetical views are disagreed with by many other investigators, the cause remains unproven and this clinical diagnosis lacks objective basis. At this stage, determination as to whether senile osteoporosis represents only one aspect of the physiological aging process or is independent pathological entity seems to be of definite clinical interest.

In this study total serum hexosamine content in normal subjects was found to increase with advancing age. Especially, the ratio by weight of glucosamine to galactosamine in the sera decreases with increasing age until fourty-nine years of age, but beyond this age limit the ratio significantly increased. On the other hand the ratio of the serum glucosamine to galactosamine was shown to be significantly low in patients with senile osteoporosis as compared with the ratio in normal group of identical age. Also, increased urinary hydroxyproline excretion in senile osteoporosis as evidenced in this study appeared to suggest collagen degradation. The present study has found evidences of abnormal mucopolysaccharide metabolism and collagen degradation in osteoporotic patients.

These observations suggest that senile osteoporosis, whatever the true cause may be, is an independent pathological entity rather than a simple result of senile metabolic decay.

Most of the studies on connective tissue have been carried out mainly on the morphological aspect, since the metabolic activity in these tissues is believed to be lower than in other tissues. Recent introduction of the collagen disease concept, however, has provoked biochemical studies of this tissue. New information has been gained on such vital physiologic processes as wound healing, calcification and aging of the bone and immune reaction. Utilization

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of the electron microscope, x-ray diffraction and isotope technique has produced clarification of the physical and chemical properties of collagen, the main constituent of connective tissue[1)~9)]. However, biochemical study of bone tissue has been limited mainly to those of the calcification process in the bone and cartilage. Investigations of the metabolic aspects of development, growth and senile changes of the bone has been very rare.

The present authors have carried out patho-histological and histochemical investigation of bone changes under the influence of amino acids and hormones[10)~12)]. In addition, biochemical studies of growth and senile changes of the bone and cartilage are currently being instituted[13)]. It has been found that hydroxyproline, one of the amino acid elements of collagen, is increasingly excreted in the urine of patients with malignant bone tumor and metabolic disturbances of the bone; and that mucopolysaccharides, a main constituent of bone matrix, shows specific alteration in the sera of patients with senile osteoporosis.

Osteoporosis, except that occurring secondarily to known pathogenesis, is termed senile or postmenopausal osteoporosis and arises from a still unknown cause. Primary osteoporosis may occur as a result of adrenal-gonadal imbalance[14)~19)] or of calcium deficiency[20) 21)]. However since these pathological views are disagreed with by many other investigators, the cause remains unproven and this clinical diagnosis lacks objective basis.

At this stage, determination as to whether senile osteoporosis represents only one aspect of the physiological aging process or is an independent pathological entity seems to be of definite clinical interest. The present authors have been engaged in the investigation of metabolism in the bone tissue and aging process of the bones and joints. The present clinico-biochemical investigation of senile osteoporosis has resulted in several interesting findings.

A) Methods

a) Determination of total serum hexosamine

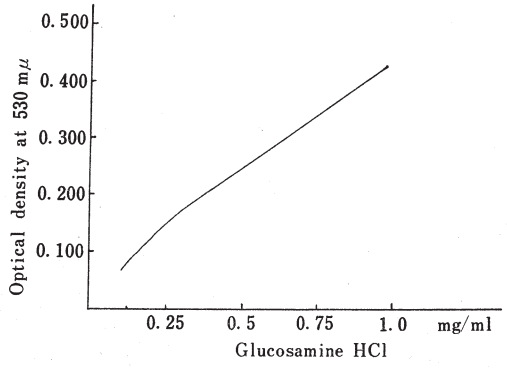
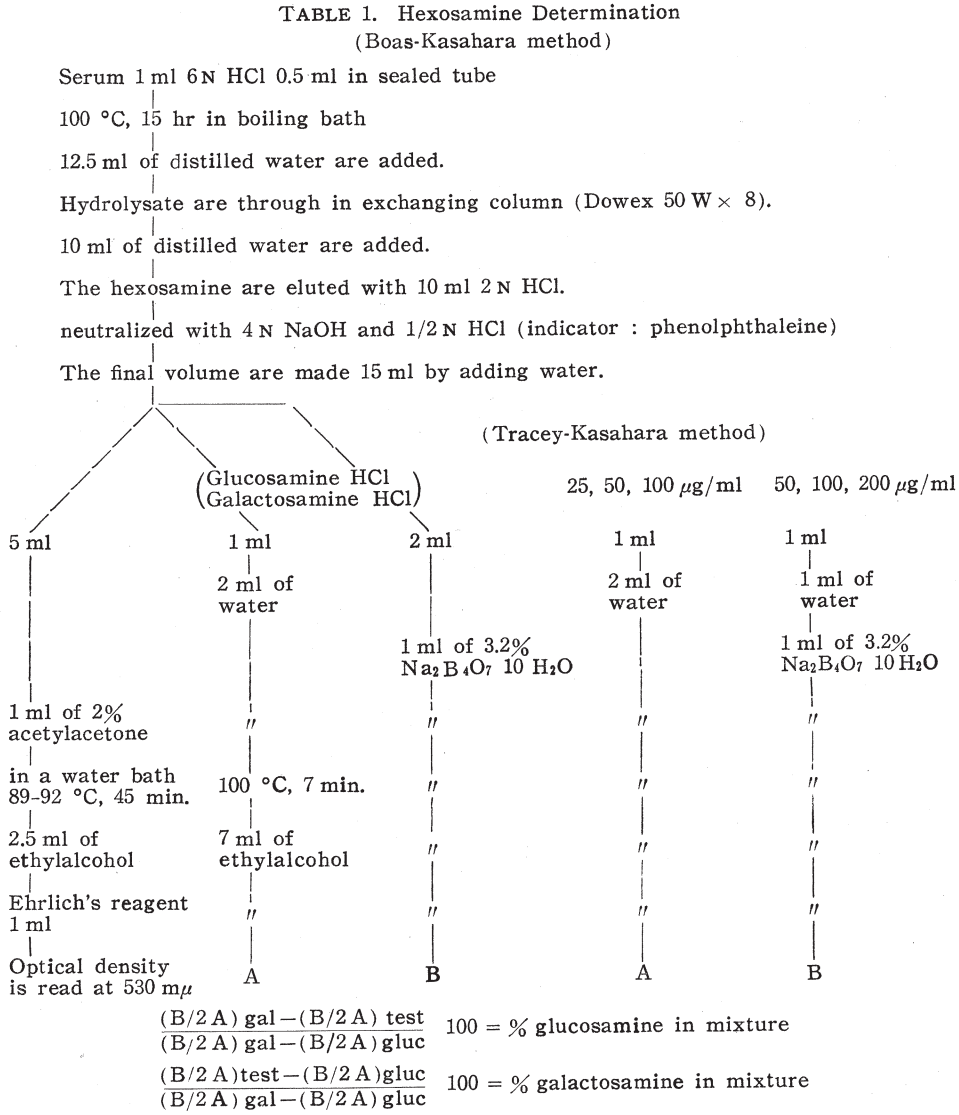


FIG. 1

Determination of total serum hexosamine was first attempted by Elson and Morgan[22)]. Although various methods have been employed since then for this purpose, they have their merits and demerits. Kasahara, one of the present authors, modified the ion exchange method of Boas[23)] and could obtain approximately a 100% recovery. In Fig. 1 the detection curve of glucosamine salt by the

Boas-Kasahara method is shown (Table 1).

TABLE 1. Hexosamine Determination



b) Determination of the ratio by weight between two amino sugars in the sera

Quantative determination of glucosamine and galactosamine may be done by one of the various methods by Slein[24], Pearson[25] or Tracey[26][27]. These methods, however, are not satisfactory when the material is serum. Kasahara has improved the Tracey method, from which he could obtain increased coloration during colorimetry. The modified Tracey-Kasahara method also enables

one to determine total hexosamine content in the sera as well as the ratio by weight of glucosamine to galactosamine in one sample (Table 1).

c) Measurement of urinary hydroxyproline

In the early stage of the present study, determination of hydroxyproline was performed according to the method described by Neuman and Logan[28]. Later, however, the method of Prockop and Udenfriend[29] was employed since this achieved a better recovery rate. During the forty-eight hours prior to the experiment all patients were placed on a diet free from fish, gelatin, ice-cream, candy and animal skins.

In the following twenty-four hours total urine was collected after the first urine in the morning was discarded. The urine was collected under toluene in a glass container and refrigerated. Hydroxyproilne is excreted in human urine in either free or bound form. The free form constitutes only four per cent of total hydroxyproline, and the remaining can be released from its bound form (polypeptide) by acid hydrolysis at 124°C for three hours.

B) Materials

a) Normal subjects in varing age groups (controls)

A total of forty-five healthy human subjects ranging from nine to seventy years of age were subjected to determination of total serum hexosamines and the ratio by weight between the two amino sugars and urinary hydroxyproline. The changes in these determinations were studied in different age groups (Table 2).

TABLE 2. Clinical Signs of Normal Subjects (Controls)

Case	Age (Yrs)	Sex	Age at Meno-pause	Radiological findings				Serum Total Hexosamine and Gluc./Galac. Ratio	
				Bone atrophy	Rarefaction of trabeculae	Vertebral collapse	Com-pression fracture	Total (mg/dl)	Glu/Gal
1	42	F	—	—	—	—	—	114.0	0.70
2	41	M		—	—	—	—	78.0	0.36
3	43	F	—	—	—	—	—	93.0	0.25
4	44	M		—	—	—	—	119.0	0.36
5	46	F	44	—	—	—	—	147.0	0.69
6	53	F	41	—	—	—	—	101.5	0.11
7	53	M		—	—	—	—	95.0	0.42
8	54	M		—	—	—	—	49.0	0.92
9	55	F	45	—	—	—	—	133.0	0.54
10	56	F	42	—	—	—	—	98.0	2.01
11	56	F	46	—	—	—	—	115.0	1.35
12	58	F	44	—	—	—	—	85.1	0.67
13	60	M		—	—	—	—	112.0	0.93
14	63	F	48	—	—	—	—	95.0	0.62
15	66	M		—	±	—	±	81.2	0.66
16	66	M		—	—	—	—	98.0	0.86
17	70	F	51	—	—	—	—	116.0	0.53
Average 59							Average:	101.9	0.70 ±0.108

TABLE 3. Clinical Signs of Senile Osteoporotic Patients

Case	Age (Yrs)	Sex	Age at Meno-pause	Radiological findings				Serum Total Hexosamine and Gluc./Galc. Ratio	
				Bone atrophy	Rarefaction of trabeculae	Vertebral collapse	Com-pression fracture	Total (mg/dl)	Glu/Gal
1	49	F	39	+	−	+	−	95.0	0.20
2	52	F	48	+	+	−	−	65.1	0.43
3	61	F	50	+	+	−	+	112.0	0.21
4	63	F	53	++	+	+	+	92.0	0.11
5	65	F	37	++	+	+	+	87.5	0.11
6	65	F	47	++	++	+	+	77.0	0.14
7	66	F	44	+	−	−	+	119.0	0.38
8	67	F	51	++	+	−	−	63.0	0.11
9	68	F	45	++	+	−	−	74.0	0.67
10	68	F	47	+	+	−	+	116.2	0.11
11	68	F	39	+	+	−	−	98.0	0.21
12	68	F	48	+	+	−	+	96.0	1.42
13	76	F	45	+	+	−	−	119.0	0.18
14	78	F	52	+	−	−	−	138.0	0.69
15	65	M		++	+	−	+	110.0	0.27
16	67	M		+	+	+	+	78.4	0.11
17	78	M		++	+	−	+	84.7	0.09
18	78	M		+	+	−	−	112.0	0.28
Average 67				18	15	5	10	96.4	0.32 ±0.13

b) Senile osteoporotic patients

A total of thirty-three male and female patients with senile osteoporosis were studied on the basis of the diagnostic criteria of Gordan[30], Barnett and Nordin[31]. Symtomatically, they all complained of back pain and all presented definite x-ray evidence of atrophy or definite deformity of the vertebral body (Table 3). They were subjected to biochemical determinations as were the

TABLE 4. Clinical Signs of Patients examined for Serum Ca, P, Alkaline Phosphatase, and Radiological Finding

Groups	Sex	No. of Cases	Average of Age (Yrs)	Serum			Radiological findings			
				Ca (mg/l)	P (mg/dl)	Alk. P-tase (Bod. U)	Bone atrophy	Rarefaction of trabeculae	Vertebral collapse	Com-pression fracture
Normal Subjects	Male	5	60	6.51	3.40	6.13	0	1	0	0
	Female	7	59	5.95	3.61	4.37	0	0	0	0
	Total or Average	12	59.5	6.23	3.51	5.26	0	1	0	0
Senile Osteoporosis	Male	4	72	5.47	3.63	4.52	4	4	1	3
	Female	14	66.5	5.31	4.11	5.60	14	11	4	7
	Total or Average	18	68.5	5.39	3.87	5 06	18	15	5	10

healthy human subjects used as controls. Before the experiment, serum calcium, phosphorous and alkaline phosphatase were determinated to exclude cases of osteomalacia or osteitis fibrosa. Questionable cases were excluded from the study (Table 4).

c) Osteoporotic patients receiving anabolic steroid for therapeutic purpose

Eight patients with senile osteoporosis receiving anabolic steroid in a daily dose of 50 to 70 mg (Total: 29,350 to 2,625 mg, mean: 11,279 mg) and calcium lactium 7 to 10 g/day (Total: 1,050 to 392 g, mean: 620, 2 g) were studied.

d) Twelve cases with osteoarthritis were noted

Fasting blood samples were taken early in the morning.

C) Results

1) Total serum hexosamine

a) Normal variations in different age groups

It is seen in Fig. 2 that total hexosamine content in the sera of normal subjects increases with advancing age of the subjects until 50 years of age; but there is no significant difference among different age groups.

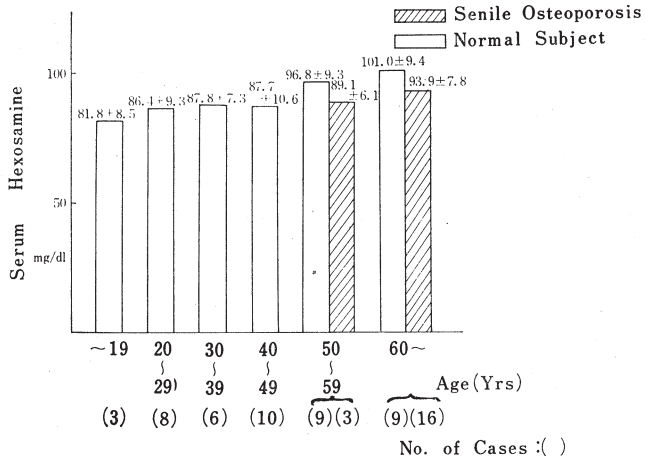


FIG. 2. Variations with age of serum hexosamine in normal subjects and senile osteoporosis

b) Osteoporotic patients

Total hexosamine content in the sera of osteoporotic patients is lower (but insignificantly so) than in the control. In patients between 50 and 59 years of age this is 89.1±6.1 mg/dl and in those aged between 60 and 70 years is 93.9±7.8 mg/dl. In the control subjects total hexosamine content is 96.8±9.3 mg/dl and 101.0±9.4 mg/dl in corresponding age groups (Fig. 2).

c) Osteoporotic patients treated with anabolic steroid

The total serum hexosamine content was not altered by anabolic steroid administration (Fig. 3).

d) Rheumatoid arthritis treated with corticosteroid

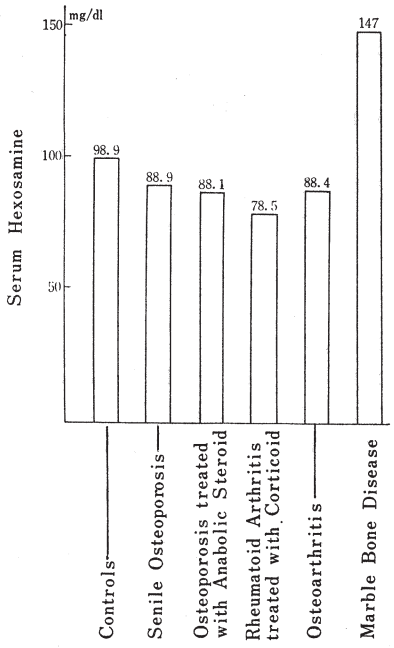


FIG. 3. Comparison of serum hexosamine in controls, patients with various bone diseases

The mean total hexosamine content is 78.5 mg/dl. This is significantly lower than in the normal and osteoporotic subjects (Fig. 3).

e) Osteoarthritis

Total hexosamine is slightly more reduced than normal but the difference is not significant (Fig. 3).

2) Changes in the ratio by weight between the two amino sugars in the sera: glucosamine to galactosamine ratio

a) Variations in the normal group according to varying ages

It is evident from Fig. 4 and Table 5 that the glucosamine: galactosamine ratio in the sera of normal subjects between 9 and 49 years of age is 0.59 to 0.47. The ratio tends to decrease with increasing age, but the variation is

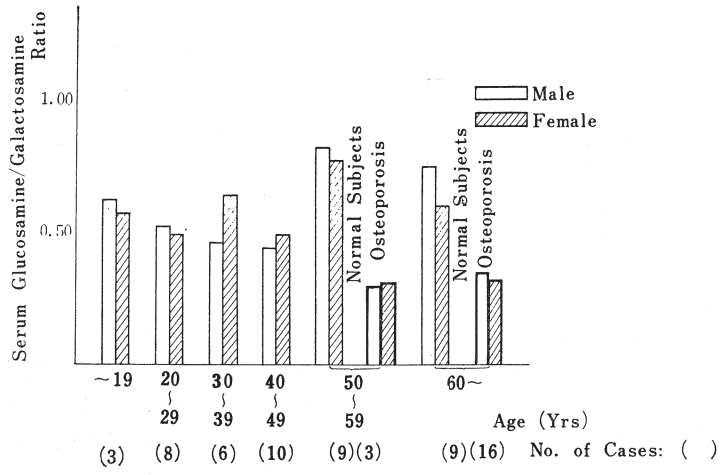


FIG. 4. Variations with age of the ratio by weight between the two amino sugars in normal subjects and senile osteoporosis

TABLE 5. Changes in the Ratio by Weight between the Two Amino Sugars in the Sera of Osteoporotic Patients

Sex	Age (Yrs)	~19	20-29	30-39	40-49	50-59		60~	
						Normal	Porosis	Normal	Porosis
Male	Gluc./Galac.	0.62	0.52	0.46	0.44	0.82	0.30	0.75	0.35
Female	Gluc./Galac.	0.57	0.49	0.64	0.49	0.77	0.31	0.60	0.27
Average		0.595	0.495	0.50	0.465	0.795	0.30	0.675	0.37

not significant. On the other hand, in subjects of both sexes between 50 and 70 years in age the ratio increases 0.80 to 0.68 with the increasing age of the subjects.

b) Osteoporotic patients

The serum glucosamine: galactosamine ratio in male osteoporotic patients was 0.30 from 50 to 59 years of age and 0.35 between 60 and 69 years of age. In the female, the ratio was 0.31 and 0.27 in the respective age groups (Fig. 4 and Table 5). The controls, male and female, averaged 0.80 and 0.68 respectively. Therefore the ratio of the serum glucosamine to galactosamine was shown to be significantly lower in patients with senile osteoporosis as compared to that of the controls in the identical age group. Also, it has been found that in fifteen patients with senile osteoporosis serum total hexosamine averaged 108.4±16.2 mg/dl, which was somewhat higher than the normal mean of 103.8±15.2 mg/dl. On the other hand, the mean glucosamine: galactosamine ratio was 0.34 in osteoporotic patients as compared to 0.58 in the normal, the difference being significant (Table 6, Fig. 5).

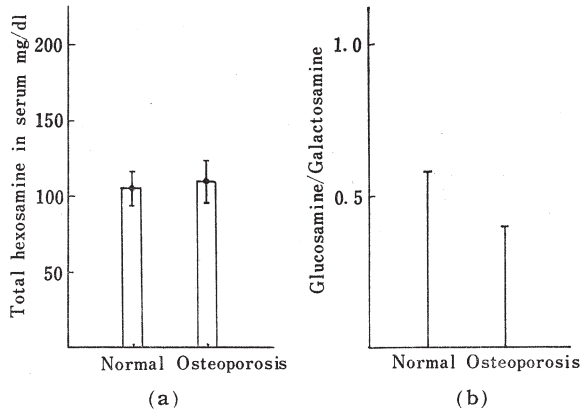


FIG. 5-a. Comparison of serum total hexosamine content in patients with senile osteoporosis and normal subjects

FIG. 5-b. Comparison of serum glucosamine/galactosamine ratio in patients with senile osteoporosis and normal subjects

TABLE 6. Comparison of Serum Total Hexosamine and Glucosamine/Galactosamine Ratio in Patients with Senile Osteoporosis and Normal Subjects

Groups	No. of cases	Total hexosamine in serum (mg/dl)	Glucosamine/ Galactosamine
Normal subjects	10	103.8±15.2	0.58
Senile osteoporosis	15	108.4±16.2	0.34*

* There was a significant difference by means of t-test ($p = 0.05$).

c) Osteoporotic patients receiving anabolic steroid

Pre-treatment glucosamine: galactosamine ratio in eight osteoporotic patients was 0.33. The ratio increased to an approximately normal level of 0.65 after administration of anabolic steroid (Fig. 6, Table 7).

d) Rheumatoid arthritis patients receiving corticosteroids

In these patients total serum hexosamine was markedly reduced. Also the glucosamine to galactosamine ratio was significantly reduced with a mean of 0.24 (Fig. 6, Table 7).

e) Osteoarthritis

The ratio of glucosamine to galactosamine in the sera averaged 0.67 and was approximately on the normal level (Fig. 6, Table 7).

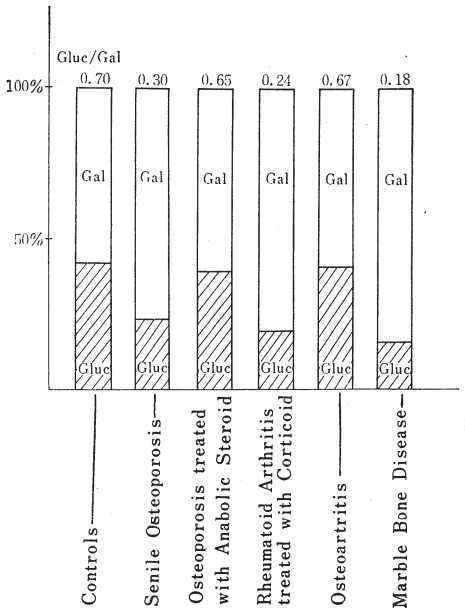


FIG. 6. Comparison of the ratio by weight between the serum amino sugars in normal subjects and various bone diseases

TABLE 7. Comparison of the Ratio by Weight between the Serum Amino Sugars in Normal Subjects and Various Bone Diseases

Senile Osteoporosis		Osteoporosis treated with Anabolic Steroid		Rheumatoid Arthritis treated with Corticoid		Osteoarthritis		Marble Bone Disease	
Gluc.	Galac.	Gluc.	Galac.	Gluc.	Galac.	Gluc.	Galac.	Gluc.	Galac.
25.0	75.0	39.4	60.6	19.2	80.8	40.3	59.7	15.5	84.5
Gluc./Galac. 0.33		Gluc./Galac. 0.65		Gluc./Galac. 0.24		Gluc./Galac. 0.67		Gluc./Galac. 0.18	

Summary of the chapter

The pathophysiological significance of mucopolysaccharides in the bone tissues is little known, although they constitute a considerable portion of bone matrix and show specific properties.

Winzler[32] found an increase of serum glycoprotein in neoplastic growth, diabetes, pregnancy, infections like tuberculosis and rheumatic fever, burns and fractures. He indicated that increase of alpha-globulin is a freqently associated phenomenon. Serum hexosamine is known to increase in patients with active tuberculosis, subacute bacterial endocarditis, parenchymatous liver disease and advanced cancer. In these cases except in liver disease, amino sugars in glycoprotein are believed to increase simultaneously and equally.

In this study total serum hexosamine content in normal subjects was found to increase with advancing age. Especially, the ratio of glucosamine to galactosamine decreases with advancing age until 49 years of age, but beyond this age limit the ratio significantly increased. On the other hand the ratio of the serum glucosamine to galactosamine is shown to be significantly low in patients with senile osteoporosis as compared with the ratio in the normal group of identical age. To be more detailed, the serum galactosamine content increased in the ratio by weight. It is also evident that the decrease in the ratio in osteoporotic patients returned to approximately a normal level after administration of anabolic steroid. The fact that mucoprotein in the bone and cartilage alters with different ages of the subjects is suggested by Kuhn[33] and Stidwoorthy[34].

In 1962, Casuccio[35] found a mucoprotein decrease in the protein content in senile vertebral spongiosa. Bertolin and Greco[36] reported increased urinary excretion of mucoprotein in senile subjects.

Increase of serum galactosamine in ratio by weight in osteoporotic patients as revealed by this study theoretically coincides with its decrease in the bone (Casuccio) and increase in the urine (Bertolin). These observations suggest a series of metabolic disorders of glycosaminoglycans in the bone, serum and urine.

By utilizing low calcium diet, experimental osteoporosis was produced in

rats according to the Nordin theory. The present authors also found that the glucosamine: galactosamine ratio increased in the bone and decreased in the serum of these osteoporotic rats. In these animals the changes in the bone matrix are thought to be similar to that in human osteoporosis, since hexosamine content in the bone is decreased and hydroxyproline content in the bone is increased.

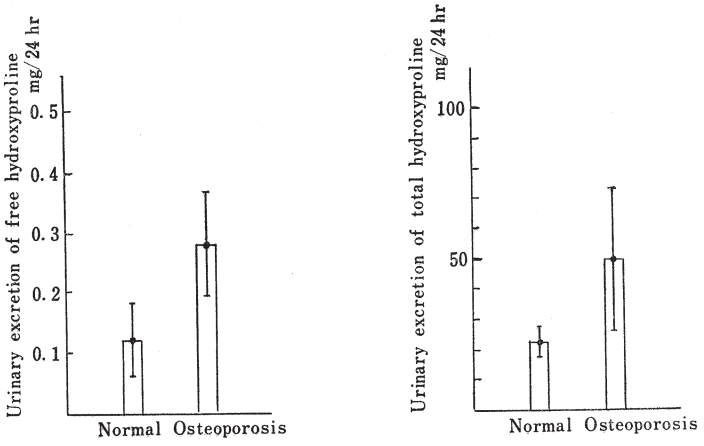
The marked decrease in total serum hexosamine content and amino sugar ratio in patients with rheumatoid arthritis receiving corticosteroids may be caused by the corticosteroid, since serum hexosamine content in rheumatoid arthritis without corticosteroid treatment is reported to be increased.

As a mechanism the significance in physiology and pathology of "*in vivo*" metabolism of glycosaminoglycans is not clearly known, and the present observation in osteoporotic patients may be of considerable significance.

3) Changes of urinary hydroxyproline

In a previous paper by the same authors[13) 37)] a marked increase in urinary total hydroxyproline excretion occurring in various pathologic conditions as malignant bone tumor, hyperparathyroidism, and in rats receiving parathyroid extract and congenital systemic bone disease was discussed. As in the previous study, free hydroxyproline was not subjected to detailed study because of its minor per cent content (4%) in total hydroxyproline. In the present study of senile osteoporosis, urinary excretion of this amino acid in free and bound form was followed up.

It has been found that in eight patients with senile osteoporosis urinary



(a) (b)

FIG. 7-a. Comparison of hydroxyproline (free form) excretion in patients with senile osteoporosis and normal subjects

FIG. 7-b. Comparison of total hydroxyproline excretion in patients with senile osteoporosis and normal subjects

TABLE 8. Comparison of Hydroxyproline Excretion in Patients with Senile Osteoporosis and Normal Subjects

Groups	No. of cases	Urinary excretion of total hydroxyproline (mg/24 hr)	Urinary excretion of free hydroxyproline (mg/24 hr)
Normal Subjects	5	22.8±4.32	0.12±0.06
Senile Osteoporosis	8	49.7±24.4	0.281±0.09*

* There was a significant difference by means of t-test ($p = 0.05$).

total hydroxyproline (polypeptide) excretion averaged 49.7±24.4 mg/24 hr, which was somewhat higher than the normal mean of 22.8±4.32 mg/24 hr, although the difference was statistically insignificant.

On the other hand, the mean urinary free hydroxyproline excretion was 0.281 ± 0.09 mg/24 hr in osteoporotic patients as compared to 0.12 ± 0.06 mg/24 hr in the normal group, the difference being significant (Fig. 7 and Table 8). The significance of free hydroxyproline is not yet wholly clarified. The recent reports[38)39)] of hereditary hydroxyprolinemia with dwarfism and mental retardation suggest an increase of free form hydroxyproline in the blood and urine and a congenital absence of hydroxyproline oxidase may account for its pathogenesis.

Discussion and Summary

Osteoporosis, symptomatically characterized by bone atrophy and back pain, is defined as a condition causing rarefaction of bone trabeculae with balanced organic and mineral constituents in the bone matrix in a different form from that of osteomalacia or osteitis fibrosa.

Osteoporosis, except that occurring secondarily to known pathogenisis, is termed senile or postmenopausal osteoporosis and arises from a still unknown cause. Primary osteoporosis may occur as a result of adrenal-gonadal imbalance or of calcium deficiency. However since these pathogenetical views are disagreed with by many other investigators, the cause remains unproven and this clinical diagnosis lacks objective basis.

In 1961 Nordin[20)21)] reported that chronic calcium deficiency due to low salt diet and impaired calcium absorption and hypercalciuria is the cause of this disease. Later a considerable amount of clinical investigations were published, which in agreement found a latent negative balance of calcium in osteoporosis. On the other hand some investigators found that in some instances atrophy of the bone could not be evidenced although they had definite negative calcium balance, and it was suggested that calcium deficiency alone could not cause osteoporosis. Casuccio[35)] reported in 1962 on the basis of chemical analysis of vertebral spongiosa in varying aged human subjects that galactosamine content is reduced in the vertebral spongiosa of osteoporotic cadavers. Bertolin

and Greco[36] indicated that in patients with osteogenesis imperfecta and osteoporosis, urinary polysaccharides were increased and the ratio by weight of galactosamine to glucosamine was elevated as compared with normal subjects in the identical age group.

Kasahara, one of the present authors, was able to modify the Boas method in hexosamine determination, by which approximately 100% yield was achieved. He also modified the Tracey method that has the merit of determining the ratio by weight of constituting amino sugars in a single sample, and obtained excellent results. Utilizing the Boas-Kasahara method and Tracey-Kasahara method, total hexosamine and the ratio by weight between the two amino sugars, the glucosamine: galactosamine ratio were found to differ significantly between the sera of normal subjects and that of senile osteoporosis.

It was found that the glucosamine: galactosamine ratio in normal subjects decreased gradually with increasing age of the subjects under 49 years of age, but the ratio increased rapidly thereafter. This is contrasted with the observation in senile osteoporosis in which case the glucosamine: galactosamine ratio in the sera is significantly decreased as compared with the ratio in the identical age group of normal subjects. The decrease of the ratio by weight of glucosamine: galactosamine is caused by the significant increase in the ratio by weight of galactosamine. This agrees with the observation of Casuccio who found a galactosamine content decrease in the vertebral spongiosa of osteoporotic cadavers as well as with Bertolin and Greco, who indicated an increase of the ratio by weight of galactosamine to glucosamine in the urine of osteoporotic patients. Also correlated changes of glycosaminoglycans in the bone, sera and urine could be experimentally reproduced in a low calcium diet induced osteoporotic animals.

Mucopolysaccharides, which term is applied to a large group of polymers like hexosamines, glucosamine, galactosamine and glucuronic acid, have been classified by Meyer[40,41] and Kuhn[33], and their chemical properties and significance in physiology are being clarified. In relation to the bone and cartilage, mucopolysaccharides have been extensively studied in association with the calcification mechanism[42~53]. It is known that in these tissues mucopolysaccharides vary in content and constituents in different age groups[33,34]. However little is known about the metabolism and patho-physiological significance of mucopolysaccharides in various bone diseases. Recent electron microscopic studies[54] utilizing H^3-proline revealed that one hour after injection uptake of the isotope is evident in the chondrocyte of experimental animals and three hours after injection the radioactive substance is demonstrable in the bone matrix.

Urinary excretion of hydroxyproline, which constitutes thirteen per cent of total amino acid content of collagen, is found to vary with collagen synthesis or degradation[13,55~62].

The increased urinary excretion of hydroxyproline in osteoporotic patients as evidenced in this study appears to suggest collagen degradation.

The present study has found evidences of abnormal mucopolysaccharide metabolism and collagen degradation in osteoporotic patients. These observations suggest that senile osteoporosis, whatever the true cause may be, is an independent pathological entity rather than a simple result of senile metabolic decay.

In a more recent study[63] a change in transparency of the dermis is found frequently in patients with osteoporosis, and thereby osteoporosis is suggested to arise from the generalized connective tissue disturbances due to altered mucopolysaccharide metabolism.

Further study on the metabolism of mucopolysaccharides in the bone, sera and urine in growing and senile organisms is currently being carried out by the present authors.

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