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**BIOCHEMICAL STUDIES ON SO-CALLED OSTEOMALACIA
(*OSTEODYSTROPHIA FIBROSA*) IN HORSES · I.
THE CHEMICAL COMPOSITION PER UNIT-VOLUME
OF THE BONE**

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The osteodystrophic disease of domestic animals observed in Japan has not only had economic consequences for the animal husbandry industry, but it has presented many interesting problems for study in the field of bone pathology. The author has made on the pathochemical studies of the bone on so-called *osteomalacia* in horses, which are studies in the pathological investigations on the osteodystrophic diseases of domestic animals which have been carried on by Professor S. YAMAGIWA.

The object of this first division has been primarily to finding a method for evaluating bone composition, which is basic to this kind of research. A well-established method has yet to be developed. E. RUTISHAUSER and MAULBETSCH have stated, "Die Abschätzung des Calcium-Phosphorgehaltes des Knochens in Schnittpräparaten ist sehr mühsam und nur in Extremfällen von Knochenleiden mit Mineralschwundmöglich. Diese Schwierigkeit beton Herr Professor ASKANAZY und auch M. B. SCHMIDT geht die gleichen Gedankengänge.....".

Upon review of the literature concerned with the chemical analysis of the diseased bone it was found to be scant and the results unsatisfactory, except for experimental studies under controlled laboratory conditions (where change of bone is made evident) using small animals. In comparison, the change of bone composition in spontaneous cases found in humans and in domestic animals is usually slight, therefore conclusions resulting from studies of spontaneous cases vary; compare table 4.

In the present study, both chemical analysis and histopathological observation of the bone were made in order to observe its pathogenesis. The materials for investigation were gathered indiscriminately from slaughterhouses in various districts. They included bone in different stages of illness, of varying ages, and from different districts as growth environment was considered influential. Due

to these factors, the author considered the clarification of the pathogenesis by the common method of chemical evaluation, i. e., by the percentage composition of the bone, to be difficult and inadequate.

It is well known that there is a complete difference in the pathological and physiological condition of the hard (e. g. bone) and soft (e. g. muscle, liver) tissues. For these reasons, the present paper has shown that if the bone compositions were evaluated as per unit-volume and not by percentage composition, the qualitative findings of the pathological condition which have hitherto been difficult to ascertain can be more easily discerned.

The present paper is a discussion of this method of evaluating bone composition by per unit-volume rather than by chemical percentage.

MATERIALS

Investigations have been made on a total of 178 cases (101 from Asahigawa, 57 from Sapporo, and 20 from Obihiro) indiscriminately obtained at the slaughterhouses as shown in table 1.

TABLE 1. *Materials Subjected to Investigation*

DATUM	MATERIAL NO.	NO. OF CASES	LOCATION OF COLLECTION
June~July (1955)	Ch. 1~101	101	Asahigawa
Dec. (1955)~Mar. (1956)	Nos. 292~360*	57	Sapporo
Jan.~Feb. (1956)	Nos. 361~380	20	Obihiro
Total		178	

* : Nos. 299, 315, 325, 327~333, 343 and 350 were subjected only to histological observation.

Recently there have been several published reports on the osteodystrophic disease in which the relation between the histological and chemical changes was studied. For example, HIGGISON and WALKER and ARVIDSSON made studies on rickets and kwashiorker in human, using the fifth rib removed just proximal to the costochondral junction for histopathological and chemical examination. NAKAJIMA et al. determined the bone composition of all limbs using the albino rat. In this case the costochondral junction of the rib was examined histologically, and the limbs were examined chemically.

Osteodystrophia usually affects the entire skeleton, but the pathological conditions of the various parts of the skeleton show considerable degrees of difference. So it is desirable that the materials employed for chemical analysis and histological examination be obtained simultaneously and from bone which is easily affected by the disease so that its pathogenesis can be more easily studied.

In this investigation, chemical analyses and histological examinations were made

from transverse slices 3 to 4 mm thick of (1) *os nasale* at the distal end of the *crista facialis* and of (2) *metacarpus* at diaphysis. The samples of *os nasale* used for this study, however, were taken from horses kept in rice-paddy areas (the disease is assumed to have a tendency more easily to affect animals in rice-paddy areas rather than animals in field crop areas); also, selections were made from horses between the ages of 4 and 14 years to avoid the influences of the age factor, which is considered to have an effect on the degree of bone salt deposition.

The pathological condition of the bone is classified according to the stage of pathogenesis, for example, as done by YAMAGIWA and SATOH. From these classified groups, 11 samples each were abstracted for this analysis at random from advanced cases in which many "progressive" large-sized hole formations were macroscopically visible (Figs. 3 & 4) and from the slight or almost normal group in which "silent" small-sized hole formation could be found only by microscopic examination (Figs. 1 & 2).

The analytical data evaluated by per unit-volume (g/100 cc) and percent (%) were compared statistically.

ANALYTICAL METHODS

In order to make histopathological observations through the microscope, bone materials were fixed in 10% formalin, decalcified in 5% HCl using the electric decalcifying method and then embedded in celloidin and stained by hematoxylin-eosin.

Even though the usual methods adopted for bone analysis are those developed by CHICK, KRAMER and HOWLAND, and BAKER et al., the author took the following steps: (1) after the adhering fat, muscle, periosteum and cartilage were removed, fresh samples of *os nasale* were cleaned with saline solution; after the excess solution was removed, (2) the samples were immediately weighed and then dried for 24 hours at 110°C. The total water was found by subtracting the dry weight from the wet. Dry materials are then usually defatted and demarrowed before analysis. However in the present method, this was not required, since compositions were evaluated by per unit-volume and the materials were collected from parts with as little marrow as possible; i. e., *os nasale* or from those easily separated, i. e., *metacarpus*. (3) Specific gravity of dried materials was measured in order to evaluate the composition per unit-volume; these materials were weighed in distilled water at 15°C by hanging each sample with a fine string. The calculation was as follows:

$$\text{s. g.} = \frac{(A)}{(A) - (B)}$$

s. g.: Specific gravity of the dried bone.

(A): Weight of the dried bone (g).

(B): Weight of the dried bone in water (g).

(4) Duplicate analyses were carried out on the sample which was powdered in an iron mortar after redrying. (5) The estimations of inorganic substances were made on a filtrate which was digested overnight in 10% trichloroacetic acid (TCA) and filtrated. (6) An aliquot of the filtrate was taken for phosphorus analysis by the method described by ALLEN. (7) The mixed content of calcium and magnesium was estimated by titration using ethylenediamine tetra-acetate (EDTA) which was recommended by SCHWARZENBACH et al.; viz., 4 g/dl EDTA titrated with calcium standard solution was added to other part

of the filtrate and then an excess of EDTA was reverse titrated with N/40 $MgCl_2$ solution, at pH 10 obtained by ammonia-buffer and using Eriochrome Black T (EBT) as an indicator. (8) Magnesium was estimated by using titan yellow according to the method recommended by ORANGE and RHEIN; viz., first, abundant calcium ion in the rest of the filtrate was precipitated with ammonium oxalate at pH 6, and with the supernatant free from the precipitate, magnesium was estimated colorimetrically using polyvinyl-alcohol (PVA) as protective colloid. The amount of calcium was found by measuring the difference between the two contents; the latter (Mg) was subtracted from the former (Ca+Mg). (9) The amount of total nitrogen was estimated by using the micro-Kjeldahl apparatus on the clear digests after the powdered bone was transferred to a Kjeldahl-flask and digested by the established method. (10) In addition, the bone ash was determined by established method.

Bone compositions per unit-volume (g/100 cc) were calculated by multiplying the results of analyses by the specific gravity.

RESULTS

The macroscopical and microscopical preparations of the bone tissue representing two groups were shown in the plate.

Figs. 1 & 2 ("silent" small-sized hole formation) show normal findings both of lamellar construction and Haversian canal system; however, microscopical small-sized holes were detected frequently in the tissue (Fig. 1). In these small-sized holes (Fig. 2) there were only few cells, the protoplasm of giant cells found occasionally were rather more eosinophile and their nuclei presented pycnosis and shrinkage, and the lining with osteoblastic layer was rarely observed; in summary, it might be said that these findings have shown generally the form of "silence".

On the other hand in "progressive" large-sized hole formation (Figs. 3 & 4), many large holes in the bone tissue were observed in Haversian canal system and in other parts. In the various large-sized holes, a congestion of blood vessels, and a proliferation of fibrous tissue were macroscopically visible on the fresh materials (Fig. 3). Reticular structure and pale-nuclear cells (which are first observed in the early stages of bone destruction) had proliferated in the focus and active giant cells with light stained nuclei or linings of osteoblastic cells were observed under the microscope (Fig. 4). But in these occasions also appositions of osteoid tissue were seldom.

The results of chemical estimations (Table 2) of each of these two groups (11 of slight group and 11 of advanced group) showing distinct differences in their histological and macroscopical findings, even after some factors thought to have influence on the analytical data were taken into account, indicate that there are variations in individual differences. Though ash, phosphorus and calcium were reduced slightly in the advanced group, comparison between the percentage composition of the dried bone of these two groups has not shown any significant statistical difference (Table 3).

In comparison, however, with compositions per unit-volume (g/100 cc) which were to be made by multiplying the same analytical data by the specific gravity, the reduction of these three compositions (ash, calcium and phosphorus) is seen to have become more distinct in the advanced group and to show a significant statistical difference (Table 3).

TABLE 2. Bone Compositions Evaluated by Percent (%) and per Unit-Volume (g/100 cc) in Slight and Advanced Cases

HISTOLOGICAL CHANGE	SUBJECT NO.	SEX	AGE (Yr.)	PERCENTAGE IN DRIED BONES					GRAM PER UNIT-VOLUME OF BONES					
				S. G.*	Ash	P	Ca	Mg	Total N	Ash	P	Ca	Mg	Total N
"Silent" small-sized hole formation (slight cases)	Ch. 11	G.	8	1.51	57.0	9.9	22.6	0.13	4.62	86.1	15.0	34.2	0.20	7.00
	Ch. 24	F.	13	1.64	62.5	10.5	24.6	0.11	4.85	102.5	17.2	40.3	0.18	7.95
	Ch. 45	F.	8	1.61	57.6	10.3	24.3	0.10	5.10	92.7	16.1	39.2	0.16	8.20
	Ch. 58	F.	11	1.71	62.0	9.6	23.8	0.24	4.61	106.0	17.6	40.7	0.41	7.88
	Ch. 73	F.	12	1.66	62.6	11.3	26.2	0.10	5.00	104.0	18.8	43.5	0.16	8.30
	Ch. 86	G.	9	1.58	60.2	10.0	25.0	0.22	5.50	95.1	15.8	39.5	0.31	8.70
	Ch. 92	M.	8	1.42	58.2	9.7	23.4	0.13	5.10	82.6	13.7	33.2	0.18	7.25
	295	F.	9	1.75	62.0	10.2	24.3	0.08	4.93	107.0	17.8	42.5	0.14	8.62
	307	M.	7	1.58	55.8	9.2	21.8	0.11	4.78	83.0	14.5	34.5	0.17	7.56
	356	G.	14	1.83	50.0	8.1	19.8	0.10	4.38	91.5	14.8	35.2	0.18	8.02
358	F.	13	1.59	53.6	9.4	23.1	0.02	4.86	85.2	14.9	36.7	0.03	7.70	
Mean				1.63	58.3	9.8	23.5	0.12	4.89	94.2	16.0	38.1	0.19	7.93
"Progressive" large-sized hole formation (advanced cases)	Ch. 6	F.	13	1.31	51.0	9.1	21.8	0.13	5.45	66.9	11.9	28.6	0.17	7.15
	Ch. 22	G.	5	1.44	59.0	10.4	23.3	0.19	5.10	85.0	14.4	33.6	0.24	7.35
	Ch. 37	F.	8	1.27	59.0	9.45	21.5	0.19	5.26	74.9	11.3	27.3	0.24	6.68
	Ch. 40	G.	8	1.26	54.0	9.9	22.0	0.15	5.60	68.0	12.5	27.7	0.19	7.05
	Ch. 48	F.	6	1.51	58.0	10.1	24.6	0.17	4.92	87.6	15.3	37.1	0.26	7.43
	Ch. 52	M.	8	1.44	56.6	9.9	22.8	0.13	5.40	81.5	14.2	32.8	0.19	7.80
	Ch. 60	G.	4	1.31	55.0	9.7	22.7	0.19	5.58	72.1	12.7	29.7	0.24	7.30
	Ch. 64	G.	10	1.30	50.4	8.8	21.4	0.14	4.80	65.6	11.4	27.8	0.18	6.20
	Ch. 66	M.	9	1.16	59.4	9.2	21.8	0.22	5.90	68.9	10.7	25.3	0.25	6.84
	Ch. 88	G.	11	1.50	58.6	9.9	23.7	0.11	5.30	88.0	14.8	35.6	0.16	7.95
345	G.	13	1.25	57.5	10.1	21.7	0.18	5.67	71.9	12.6	27.1	0.20	7.10	
Mean				1.34	56.2	9.7	22.4	0.16	5.36	75.5	12.9	30.2	0.21	7.17

* : Specific gravity of dried bone (mass).

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TABLE 3. Comparison between Mean Values of Analytical Results Given in Table 1

COMPOSITION	PERCENTAGE IN DRIED BONE			GRAM PER UNIT-VOLUME OF BONE (g/100 cc)		
	Slight Cases ("Silent" small-sized hole formation)	Advanced Cases ("Progressive" large-sized hole formation)	Statistical Analyses	Slight Cases ("Silent" small-sized hole formation)	Advanced Cases ("Progressive" large-sized hole formation)	Statistical Analyses
S. G. *	1.63	1.34	P < 1% Signif.	.	.	.
Ash	58.30	56.20	P > 5% Non-signif.	94.20	75.50	P < 1% Signif.
P	9.80	9.70	P > 5% Non-signif.	16.00	12.90	P < 1% Signif.
Ca	23.50	22.40	P > 5% Non-signif.	38.10	30.20	P < 5% Signif.
Mg	0.12	0.16	P > 5% Non-signif.	0.19	0.21	P > 5% Non-signif.
Total N	4.89	5.36	P > 5% Non-signif.	7.98	7.17	P = 5% Signif.

* : Specific gravity of dried bone (mass).

Magnesium has not shown a significant difference in either percentage or per unit-volume, but there is a slight increase in the advanced group (Table 3). Total nitrogen was increased in percentage composition in the advanced group, but on the contrary, the total nitrogen was reduced significantly when computed by per unit-volume (Table 3). This finding seems to present many interesting problems.

DISCUSSION

Some biochemical studies concerned with the pathogenesis of *osteodystrophia* have been made on rickets in humans and in animals, especially experimental studies, but rather few studies have been carried out on other *osteodystrophia*. In addition, there is the tendency for even *osteodystrophia* of various kinds in adult animals to be treated in the same manner as rickets, of which there is some understanding. The cause of failure in such treatments might be attributed in part to the difficulty of the evaluation method of the bone composition, as has already been indicated by the quotation from RUTISHAUSER and MAULBETSCH. In table 4, though HUPPERT and also CAPPEZZUOLI indicate that calcium in bone ash is reduced in human *osteomalacia*, RUTISHAUSER and MAULBETSCH do not indicate the reduction of calcium and phosphorus in human *osteomalacia* and *osteoporosis*. The results of WALKER and ARVIDSSON, and HIGGISON (Table 4) on human bone from South African Bantu subjects habituated to a high cereal diet showed that the growth depression or rickets change of the bone could be observed histopathologically in spite of the fact that there was no reduction in inorganic substances. They also obtained the same results in their experiment with rats, fed on a high cereal diet. However, NAKAJIMA et al.¹⁰⁾ have shown that calcium and phosphorus in the dried bone are reduced when they used the same experiment with rats. Thus the author considers that the results of the diseased bone analyses are rather diverse.

Biochemical studies of the bone on so-called *osteomalacia* of horses which is the present subject have rarely been made. For example, HORI and NIWA have found an increase of nitrogen, and NAKAJIMA et al.¹¹⁾ carried out the bone analysis of inorganic substances of the diseased bone but had little to report. Thus, as far as the present writer is aware, results of studies on bone diseases as a whole have been unsatisfactory and scarce; the same is the case for so-called *osteomalacia* in horses. Two probable reasons for the scarcity of studies in this field and for the diverse results from accepted methods of analysis may be as follows:

Specific gravity of bone: The bone, as hard tissue, in animal body is completely different in structure and composition from soft tissue. Especially in its composition, the substance contained most abundantly is bone salt (50~60%), and the

TABLE 4. *Percentage Compositions of the Bones*

INVESTIGATORS	BONE DISEASE	SUBJECT MATERIAL		COMPOSITIONS IN BONE			
				Ca		P	
				*	**	Norm. Diseas.	Norm. Diseas.
HUPPERT (1867)	<i>Osteomalacia</i>	Human	?	• Decrease		•	•
CAPPEZZUOPLI (1909)	<i>Osteomalacia</i>	Human	?	• Decrease		•	•
RUTISHAUSER & MAULBETSCH (1934)	<i>Osteoporosis</i>	Human	Rib	36.0	36.00	14.7	15.20
NAKAJIMA et al. (1951)	Rickets-like	Rats	Limbs	38.9	4.33	16.4	17.80
NAKAJIMA et al. (1955)	So-called <i>Osteomalacia</i>	Equine	Frontal bone	35.7	36.40	17.2	16.08
WALKER & ARVIDSSON (1954)	Rickets	Human	Rib	•	•	•	•
WALKER & ARVIDSSON (1954)	Kwashiorker	Human	Rib	•	•	•	•
The present author	So-called <i>Osteomalacia</i>	Equine	Nasal bone	•	•	•	•

* : Bones of control or normal.

** : Diseased bone.

Note: Among bone compositions reported by NAKAJIMA et al. (1951)¹⁰⁾, data of normal bones were cited from "NAKAJIMA, T. & T. SUGITA (1950): Jap. J. vet. Sci., 12, 13".

chemical structure of this salt seems to be similar to that of apatite (particularly to hydroxyapatite). The specific gravity of apatite is within the range of 3.1~3.2; therefore, the amount of bone salt has much influence on the specific gravity of bone tissue. For example, the specific gravity of a certain case in the slight group was 1.83, as against 1.16 in the advanced group, as shown in table 2. These facts were very interesting in comparison with the specific gravity of nearly 1.0 of soft tissue. Thus extreme cases having severe mineral reduction require more volume to obtain the same weight as almost normal or slight cases.

A characteristic feature of the pathological condition: The pathogenetic process of the bone, viz., focal loosening, various-sized hole formation and enlargement of the hole and "clearance" on the wall may advance without change in its volume, as shown in the plate (cf. YAMAGIWA and SATOH). McLEAN and URIST

Reported by Various Investigators

ASH (%)	COMPOSITIONS IN DRIED BONE (%)						CONCLUSION	
	Ca		P		Mg			
Mg								
Norm. Diseas.	Norm. Diseas.	Norm. Diseas.	Norm. Diseas.	Norm. Diseas.	Norm. Diseas.	Norm. Diseas.		
• Increase	•	•	•	•	•	•	Decrease of Ca	
• Increase	•	•	•	•	•	•	Decrease of Ca	
•	•	•	•	•	•	•	No difference	
•	•	24.30	17.60	10.20	7.20	•	•	Decrease of Ca, P
•	•	21.74	20.42	10.47	7.06	•	•	Little to report
•	•	22.24	20.46	10.80	10.50	•	•	No difference
•	•	22.24	22.85	10.80	10.80	•	•	No difference
•	•	23.50	22.40	9.80	9.70	0.12	0.16	No difference

have stated that rarefaction of bone was by reduction of the weight in unit-volume. This same idea of change in unit-volume could be found in the study of RUTISHAUSER and MAULBETSCH; however, they did not obtain satisfactory results mainly because the specific gravity of bone was measured by pycnometric method on the powdered material.

For these reasons, when the pathogenesis of bone disease is studied biochemically, the author has recommended that the bone composition be evaluated by per unit-volume and not by percent, and that the bone specific gravity be measured as mass rather than as powder, except on the occasion in which information concerned with proportional constituents in the bone salt is desired.

Thus the biochemical examination might well be performed in parallel with the histological examination. The significance of changes in each of the constituents will be discussed in report II.

SUMMARY

In this country there is a disease in horses popularly called *osteomalacia* which has been known for many years. It has had economic consequences for the animal husbandry industry, and of it innumerable studies have been made. The author has studied the pathochemical changes of the disease as a part of the pathological investigation of the osteodystrophic disease of domestic animals. However, the chemical analysis of the diseased bone was found to yield few data and the results are unsatisfactory because of the difficulty in evaluating the analytical data and because of the characteristic feature of the composition and the pathological condition in bone.

1. The abundance of bone salt and its high specific gravity influence the specific gravity of bone tissue. The extreme cases of bone salt reduction require much more volume to obtain the same weight as the almost normal or slight cases. Therefore any significant differences could not be found between normal and pathological conditions by the common method, i.e., by estimation of percentage composition per same weight (cf. Tables 2~4).

2. The pathological change of the bone seems to develop without change in its volume principally as shown in the pathological finding (cf. Plate, YAMAGIWA and SATOH) and in the findings by McLEAN and URIST.

For these reasons, the author has recommended that the bone composition be evaluated per unit-volume (g/100 cc) and not by percent (%) (Tables 2 & 3), if the pathogenesis of the bone disease is studied pathochemically. Thus the slight change in the bone, having no compositional change in spite of the pathological condition as seen microscopically, can be examined biochemically by using the present method of evaluating bone compositions by per unit-volume.

The author wishes to express his gratitude to Prof. YAMAGIWA of the Department of Veterinary Pathology for his kind direction and review of this study, and to his assistant Mr. SATOH for kind cooperation.

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EXPLANATION OF PLATE

Figs. 1 & 3. $\times 5$, transvers-sectioned preparation photographed by transmitted light.

Figs. 2 & 4. $\times 100$.

Fig. 1. *Os nasale*; slight case, "silent" small-sized hole formation is observed microscopically.

Ch. 24, ♀, Age 13.

Fig. 2. Microscopical magnification of Fig. 1, "silent" small-sized hole formation, few cells and few giant cells with pycnotic nuclei in the hole were observed.

Fig. 3. *Os nasale*; advanced case, "progressive" large-sized hole formation. Ch. 37, ♀, Age 8.

Fig. 4. Microscopical magnification of Fig. 3, many large holes, abundant fibrous tissue, hyperemia, large giant cells with active phagocytic ability.

