Acta orthop. Scandinav. *40*, 708–720, 1969

Mayo Graduate School of Medicine, University of Minnesota; and Section of Surgical Research, Mayo Clinic and Mayo Foundation, Rochester, Minnesota, U.S.A.

A STUDY OF BONE FORMATION IN DOGS OF DIFFERENT METABOLIC STATES USING AUTORADIOGRAPHIC VISUALIZATION OF ^{45}Ca

VU VAN NGUYEN & JENIFER JOWSEY

Received ix.68

The evaluation of bone turnover using a bone biopsy technique is essentially a method that depends on taking a sample from a single area of the skeleton and comparing values for tissue formation and resorption with those from samples taken from the same area in another animal or individual. From the comparison, inferences can be made of the relative metabolic status of bone tissue in the two animals. Bone sampling has been used to measure bone turnover in normal persons or animals of different ages, and the information is used as a basis for comparison with abnormal material to allow characterization of the abnormality (*Barer & Jowsey* 1967, *Beck & Nordin* 1960, *Jowsey* 1966 b, *Jowsey et al.* 1965 a, *Lee* 1965, *Riggs et al.* 1965, *Smeenk* 1961, *Stanbury* 1961, *Villaneuva et al.* 1966).

The validity of such a method depends on the assumption that a sample is representative of the rest of the skeleton. Although the values obtained from a single bone sample need not be identical with the values found in that tissue throughout the body, there should be high correlation between different sites. In this way, from a single biopsy specimen, a prognosis can be made regarding the metabolic status of the rest of the skeleton. The significance of differences between values depends on the variations found in normal material and within one sample.

This investigation was supported in part by Research Grant AM-8658 from the National Institutes of Health, Public Health Service.

Read at the meeting of the Orthopaedic Research Society, Chicago, 19 January 1968.

The purpose of this experiment was to study bone formation at different skeletal sites in dogs known to have different metabolic conditions and to find out if unpredictable variations between sample sites of the same animal or variations between normal animals are small enough (when compared with real differences between groups of animals having different metabolic activities) to allow a sampling technique to be a valid method for evaluating and comparing skeletal metabolism.

MATERIALS AND METHODS

The experimental animal was the adult dog. Roentgenographic evidence of closure of the epiphyseal plate was established before the dog was included in the experiment. Three groups were studied: Group 1 consisted of five dogs that had been thyroparathyroidectomized 1 to 2 months before the beginning of the experiment (these dogs were expected to have low values for bone formation and resorption). Group 2 was the control group and consisted of five normal dogs, and Group 3 consisted of five postparturition and postlactation female dogs. (These animals were assumed to have a high bone turnover.)

Radioactive calcium (^{45}Ca) was given intravenously in a single injection, 50 to 200 μc/kg body weight, and the dogs were sacrificed 48 hours to 7 days later.

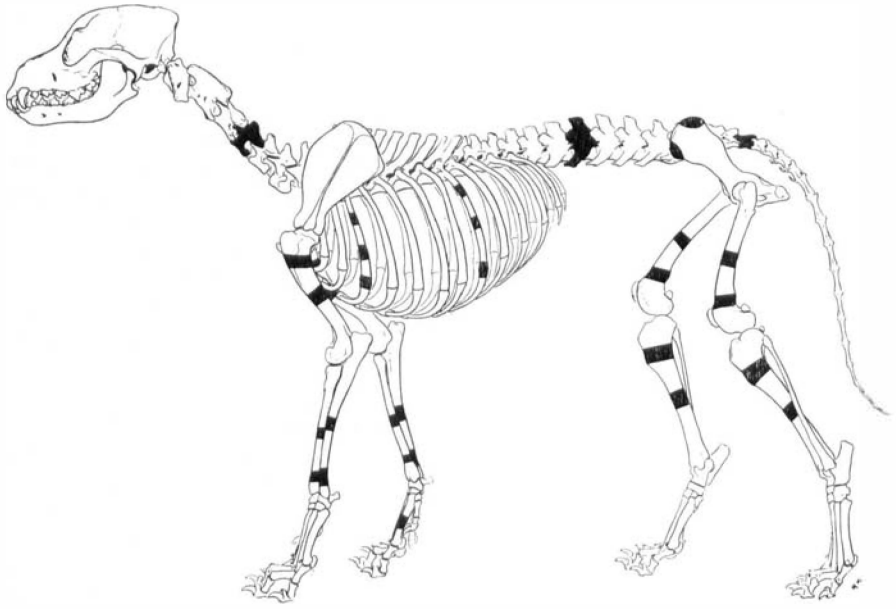


Figure 1. Skeletal sites studied in three representative dogs of different metabolic states.

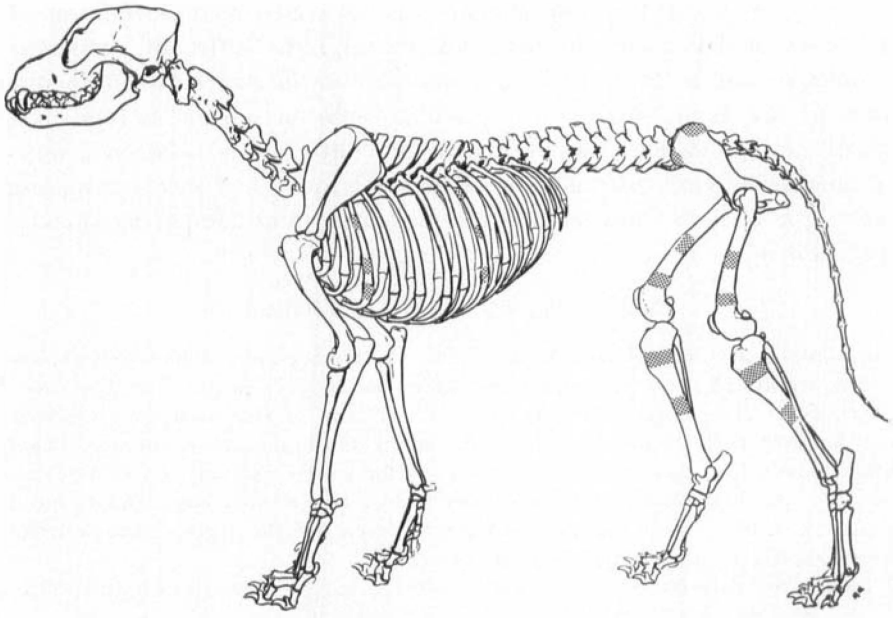


Figure 2. Skeletal sites studied in all dogs.

From one dog in each group, bone sections were taken from 33 different sites of the skeleton. These samples were taken from representative areas of the body, including both cortical and trabecular bone (spinal column, ribs, iliac crests, long bones of the anterior and posterior limbs; for details, see Appendix and Figure 1); comparable samples were taken from the left as well as the right side of the body. From the remaining animals in each group, samples were taken only from the iliac crest, rib, and the diaphysis and metaphysis from both the femur and tibia (for details, see Appendix and Figure 2). Since these sites were also studied in the three representative dogs, they were common to all animals of the three groups.

The bone samples were embedded in methacrylate, and three sections were cut from each bone sample (Jowsey et al. 1965 a). The sections were exposed on type A autoradiographic plate long enough to produce an autoradiograph of suitable density. After development, magnified prints were made of the autoradiographs, and the amount of bone formation was assessed from these pictures by measuring the areas of high uptake of ^{45}Ca. Such areas were considered to represent the calcification of new bone and therefore to represent bone formation if the density was clearly greater than that of the diffuse uptake of ^{45}Ca. Measurements were made in the following manner: The number of small osteons showing concentrated uptake of ^{45}Ca were counted; these are the so-called hot-spots. The length of all measurable surfaces with high density in the autoradiograph was assessed with a map measurer. Previous data have shown that the average perimeter of an Haversian canal in dog is 100 μ (Jowsey 1966 a). Therefore, the length of the active surface in all small osteons can be obtained by multiplying the number of hot-

spots (N) by 100 μ. This figure was added to the length of all surfaces of high specific activity (L), and the sum represented the value for the length of all surfaces in that section undergoing bone formation. The area of the bone sample (S) was then measured with a planimeter, and bone formation was expressed in terms of length per unit area of the section (mm/cm^2). Thus,

$$\text{Bone formation/unit area} = \frac{(N/10) + L}{S}.$$

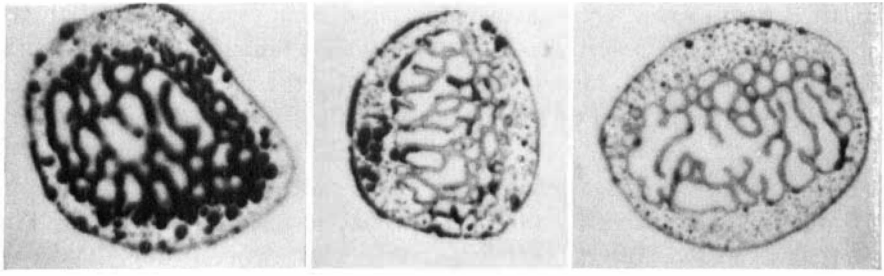
The values for the three sections were averaged, and the results between different skeletal sites in each animal, different animals in each group, and different groups were compared. The data were pooled from each similar skeletal site for computing correlations and the significance of differences; for example, iliac crest refers to values from the right and left anterior and the right and left posterior aspects of the iliac crest (see Appendix).

RESULTS

The autoradiographs of the sections of labeled bone showed distinct areas of high density. In some skeletal sites, such as the iliac crest and rib, there was an obvious difference in the number of areas of concentrated uptake of ^{45}Ca (Figure 3).

Analysis of Bone Formation in the Three Representative Dogs in Each Group

Results obtained for bone formation per unit area were first compared between the three representative dogs in each group. First, 14 different pairs of data were compared, a value from the right side and one from the left side in the same area in the bone constituting the two values in each pair. The results show a high positive correlation between such pairs (Table 1).



A B C

Figure 3. Autoradiographs of cross-sections of a rib from each of three representative dogs in each group show variation in number of hot-spots. A, Group 1, hyperactive; B, group 2, control; C, group 3, hypoactive (all × 11).

Table 1. Correlation from left to right sides in all sites in the three representative dogs

Dog	N	r	t	df
Hyperactive	14	0.920	8.133	12
Control	14	0.941	9.665	12
Hypoactive	14	0.973	14.634	12

Table 2. Bone formation per unit area in the three representative dogs*

Dog	Spinal column†	Rib†	Iliac crest†	Long bones	
				Diaphysis§	Metaphysis§
Hyperactive	116.0 ±40.0	153.0 ±10.5	142.0 ±13.0	43.7 ±2.6	57.9 ±7.6
Control	22.6 ±7.9	86.1 ±7.3	84.0 ±18.0	10.9 ±2.5	16.8 ±4.1
Hypoactive	15.8 ±5.5	22.2 ±1.3	30.4 ±5.6	6.7 ±0.7	10.3 ±0.7

* Mean ± SE.

† Values represent pooled data from all sections in this site (see Appendix).

§ Diaphysis = pooled data from femur, tibia, humerus, radius, ulna, and metatarsals; metaphysis = pooled data from femur, tibia, humerus, radius, and ulna (see Appendix).

Next, a comparison was made in these same three dogs between the amount of bone formation in different sites in the skeleton (Table 2). The rib and iliac crest consistently showed the highest activity in all three groups, whereas the long bone diaphysis, not surprisingly, showed the lowest activity in all three animals. Considerable differences were found in bone formation between the three animals; the ratio was as high as 4 to 1 between the hyperactive and the normal dog and up to 2.5 to 1 between the normal and the hypoactive dog. The t-test was applied to paired data; each pair consisted of the bone formation value in the same skeletal site in the hyperactive and in the control dog, or in the hypoactive and control dog. The test establishes whether real differences exist between the different groups of animals, whatever site was chosen. The results in Table 3 show that in all skeletal sites the hyperactive dog was significantly different from the control dog (95 and 99 per cent confidence levels). In the

Table 3. Comparisons for similarity or dissimilarity between the three representative dogs*

Skeletal site	N	Control versus hyperactive dog		Control versus hypoactive dog	
		T value	*P* value	T value	*P* value
Spinal column†	3	2.80	<.05	0.87	<.5
Rib†	4	6.03	<.01	9.93	<.01
Iliac crest†	4	3.02	<.05	3.28	<.05
Long bones					
Diaphysis§	12	9.49	<.01	1.70	<.1
Metaphysis§	10	5.01	<.01	1.68	<.1

* With control group (t-test).

† The data consist of pooled values for all sections in this site (see Appendix).

§ Diaphysis = pooled data from femur, tibia, humerus, radius, ulna, and metatarsals; metaphysis = pooled data from femur, tibia, humerus, radius, and ulna (see Appendix).

comparison between the hypoactive and the control dog, the spinal column and long bones did not show significant differences; the confidence levels were 50 and 90 per cent, respectively. However, the rib and iliac crest showed a significant difference at the 95 and 99 per cent confidence levels, respectively. At all sites, bone formation in the hypoactive dog was significantly less than that in the hyperactive dog.

Analysis of Bone Formation in All Dogs in the Three Groups

An analysis was carried out in all dogs in the three groups in which bone formation in a single site was compared with other sites in the same skeleton. The site chosen was the iliac crest. This was compared with rib and tibial and femoral metaphyses and diaphyses. The bone formation values demonstrated a small randomization frequency ($P < .01$). There was a high correlation ($r = 0.951$) between rib values and iliac crest values in the same animal (Figure 4). The bone formation values in the long bones showed a somewhat lower but, nevertheless, a high positive correlation; the metaphyses had a higher correlation with iliac crest ($r = 0.826$, femur metaphysis; $r = 0.827$, tibia metaphysis) than the diaphyses ($r = 0.780$, femur diaphysis; $r = 0.737$, tibia diaphysis).

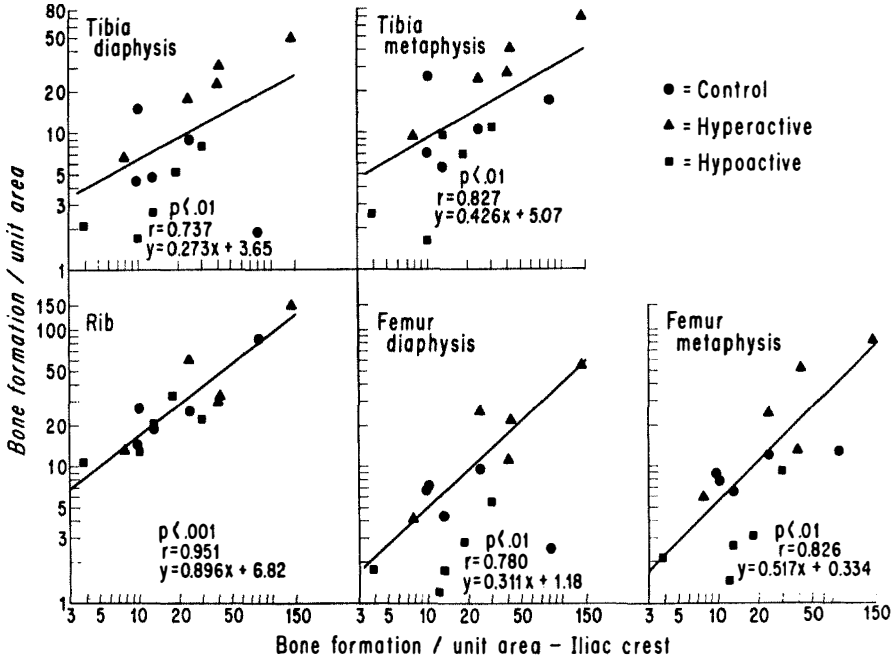


Figure 4. Correlation of bone formation at different skeletal sites in dogs in different metabolic conditions. Different sites are compared with the iliac crest. Note that data are plotted on log:log paper.

DISCUSSION

The use of ^{45}Ca as a label for the deposition of mineral in newly formed bone is now well established. Concentrated uptake of the isotope has been associated with unequivocal evidence of tissue depositions, such as the presence of osteoid and double labeling with tetracycline (*Harris et al.* 1962, *Lee et al.* 1965, *Marotti & Marotti* 1965, *Marshall et al.* 1959 a, *Marshall et al.* 1959 b, *Strandh & Bengtsson* 1961). Careful autoradiographic studies of bone from animals killed at different times after injection of the ^{45}Ca have indicated that after 24 hours the majority of concentrated isotope deposition is associated with formation, rather than short-term exchange on surfaces (*Rowland* 1966). Therefore, the basic premise of this report is valid: Concentrated deposition of ^{45}Ca occurs in areas of mineralization of new bone and linear measurements of such areas visualized autoradiographically are a measure of bone formation.

Evaluation of skeletal metabolism by biopsy methods rests on the

same assumption as many pathologic methods in which a small piece of tissue is examined and a diagnosis that refers to the whole organ is made. The skeleton is unquestionably more variable in its metabolic activity from one area to another than most organs of the body. Those variations that are found do not, however, have to be considered as sampling errors, since these variations are predictable, within reasonable limits, from one site to another. The results reported here show a good correlation between the different sites in the same skeleton ($r > 0.737$ for all sites studied). Therefore, the same areas can be compared in different skeletons; and if the relationship between the sites is known, different sites in different skeletons can be compared. Areas of low tissue turnover, such as the diaphyses of long bones, are unsuitable for evaluating conditions of low bone turnover, such as hypoparathyroidism.

The data obtained on rib material in these animals indicated that the rib is a valid site for biopsy because it showed clearly differentiated changes in different metabolic conditions that correlated well with the iliac crest. This has not been the invariable experience with measurements of bone resorption in man, particularly in older age groups, possibly because the rib contains small amounts of bone tissue and ceases to represent the skeleton after a certain amount of tissue has resorbed (*Barer & Jowsey* 1967). In other skeletal areas, the information obtained supports the conclusions derived from studies in man: That the iliac crest, containing a representative amount of cortical and trabecular bone, is the most suitable area for biopsy (*Jowsey* 1966 b), whereas diaphyseal bone is the least active and only shows difference after intensive changes in metabolism.

Previous investigations regarding the variations in bone formation in dogs have utilized the uptake of tetracycline. *Amprino & Marotti* (1964) and *Jowsey et al.* (1965 b) studied dogs ranging in age from 2 to 36 months, and they showed large variations in bone formation from animal to animal. However, the results appeared to be consistently different and therefore predictable. *Harris et al.* (1967) have shown the importance of variations in the site of sampling in growing animals, and it is evident that the error incurred in sampling, in animals that are increasing in skeletal size, or in the remodeling phase which follows the growth period is greater than in adult animals in which all bone turnover related to growth has ceased. In this context, *Amprino & Marotti's* (1964) data have indicated that a dog must be at least 18 months old before this state is achieved. In our opinion,

a dog must be 2½ years old before the skeleton is considered to be adult (*Jowsey et al.* 1965 b), and the sampling error reasonable.

Most investigators who have used the biopsy method for evaluating bone metabolism in man have been interested in metabolic changes that are large compared with the variations found in their control group. Evaluations may be merely visual estimations of porosity (*Van der sluys Veer et al.* 1964) or may be quantitative measurements of bone surfaces involved in bone formation judged by tetracycline labeling (*Lee* 1965, *Marotti & De Lena* 1966, *Villaneuva et al.* 1966), histologic methods (*Riggs et al.* 1965, *Stanbury* 1961), or microradiography (*Marshall et al.* 1959 a, *Smeenk* 1961). The last two methods supply information derived from measuring bone resorption as well as bone formation.

In this study it was possible to produce dogs with variations in skeletal metabolism comparable to those found in most human metabolic bone diseases. The results show that variations from group to group were larger than variations from site to site or from left side to right side. *Harris et al.* (1967) have reported data on three animals in which they made careful and extensive measurements of bone formation using tetracycline deposition. The animals were all normal

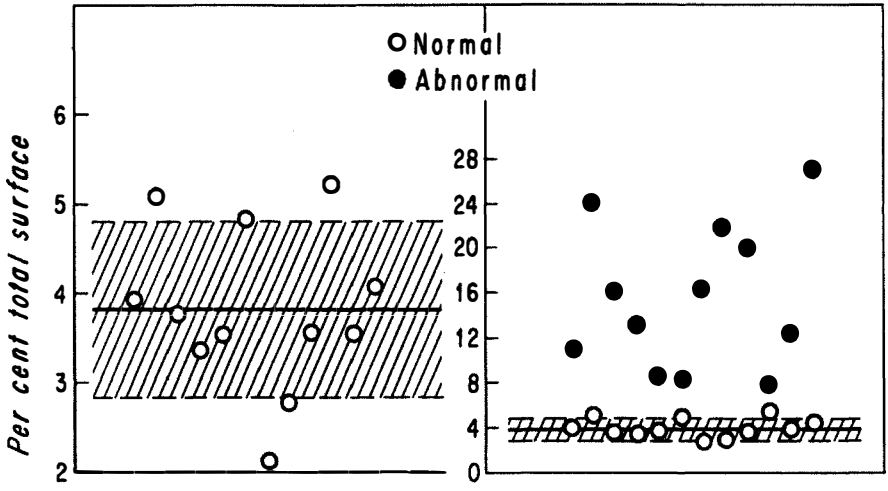


Figure 5. Bone resorption values in age-matched normal and abnormal rib biopsy specimens. Left panel shows variation found in normal material (mean ± 1 SD). Right panel shows same data plotted with data from known cases of bone-losing disease. Note that values are higher for resorption.

and demonstrated the variations to be expected in such a group. A variation was also found in the dogs reported on here. However, in order to evaluate the validity of a biopsy method, it is necessary to find out if the values for bone formation found in known metabolically different skeletons are quantitatively different from the control values. If such is true, as has been shown in this report, then a biopsy method is valid.

A particular experience with human material may serve to illustrate the point. Ribs from 12 persons with an unknown bone-losing disorder were measured by the method of quantitative microradiography. Values for age-matched normal material were compared with these (Figure 5). The normal values fall clearly below those found in the metabolically different group. Although variations in normal material may be large, they are smaller than the differences between the normal and the abnormal material.

Harris et al. (1967) also demonstrated variations in the width of new tissue deposition at different times and variations in the formation rate. The method used in this study and also the question to be answered are concerned only with the number or dimensions of bone-forming sites and not with rate.

SUMMARY

Biopsy methods are based on the assumption that a sample of tissue is representative of that tissue throughout the body. Such methods have been used to evaluate bone turnover in the skeleton. The present study was undertaken to examine this assumption: To find if the variations within individuals of a single metabolic state and the differences in the ratios between sample sites are small enough, when compared with real differences in bone metabolism, to justify the initial assumption.

Three groups of adult dogs were studied; the first group consisted of five dogs that had been thyroparathyroidectomized 1 to 2 months before the beginning of the experiment; the second group consisted of five normal dogs; and the third group consisted of five postparturition and postlactation dogs. Bone formation was quantitated by means of autoradiography with ^{45}Ca.

The results indicate that the three groups differed significantly in levels of bone formation. These differences were apparent in the skeleton of any one dog. However, each site had a relatively constant

ratio to all other sites. Any variations from the left to the right side were within the limits that distinguished one group from another. Only when bone turnover was low, as in the midshaft of long bones in thyroparathyroidectomized animals, was there a failure to distinguish between groups of different metabolic states.

ACKNOWLEDGMENTS

The authors would like to thank Dr. *B. L. Riggs* for his assistance in this project, and are grateful to Mr. *Eldon Greenwaldt* and Mrs. *Lucille M. Stucky* for their technical help.

REFERENCES

Amprino, R. & Marotti, G. (1964) A Topographic Quantitative Study of Bone Formation and Reconstruction. In *Proceedings of the first European bone and tooth symposium, Oxford, April, 1963*, p. 21. Macmillan Co. New York.

Barer, M. & Jowsey, Jenifer (1967) Bone formation and resorption in normal human rib bone: A study of persons from 11 to 88 years of age. *Clin. Orthop.* **52**, 241–247.

Beck, J. S. & Nordin, B. E. (1960) Histological assessment of osteoporosis by iliac crest biopsy. *J. Path. Bact.* **80**, 391–397.

Harris, W. H., Haywood, Edith A., Hamblen, D. & Lavorgna, J. (1967) Spatial and temporal heterogeneity of bone-formation rates. *J. Bone Jt Surg.* **49**-A, 793.

Harris, W. H., Jackson, R. H. & Jowsey, J. (1962) The *in vivo* distribution of tetracyclines in canine bone. *J. Bone Jt Surg.* **44**-A, 1308–1319.

Jowsey, Jenifer (1966 a) Studies of haversian systems in man and some animals. *J. Anat.* **100**, 857–864.

Jowsey, Jenifer (1966 b) Quantitative microradiography: A new approach in the evaluation of metabolic bone disease. (Editorial). *Amer. J. Med.* **40**, 485–491.

Jowsey, Jenifer, Kelly, P. J., Riggs, B. L., Bianco, A. J., Jr., Scholz, D. A. & Gershon-Cohen, J. (1965 a) *J. Bone Jt Surg.* **47**-A, 785–806, 872.

Jowsey, Jenifer, Lafferty, W. & Rabinowitz, J. (1965 b) Analysis of distribution of Ca^{45} in dog bone by a quantitative autoradiographic method. *J. Bone Jt Surg.* **47**-A, 359–370.

Lee, W. R. (1965) A quantitative microscopic study of bone formation in a normal child and in two children suffering from osteogenesis imperfecta. In *Proceedings of the second European symposium on calcified tissues, Domaine Provincial de Wegimont, March 31 to April 4, 1964*, p. 451. Université de Liège, Liège.

Lee, W. R., Marshall, J. H. & Sissons, H. A. (1965) Calcium accretion and bone formation in dogs: An experimental comparison between the results of Ca^{45} kinetic analysis and tetracycline labelling. *J. Bone Jt Surg.* **47**-B, 157–180.

Marotti, F. & Marotti, G. (1965) Validità dell' impiego delle tetracicline nella valutazione quantitativa della formazione e del rinnovamento del tessuto osseo. *Arch. Putti* **20**, 394–409.

Marotti, G. & De Lena, M. (1966) Analisi quantitativa dei processi di ricostruzione strutturale nella mandibola del cane in rapporto all' etta. *Arch. ital. Anat. Embriol.* **71**, 229–252, Fascicle 3.

Marshall, J. H., Jowsey, J. & Rowland, R. E. (1959 a) Microscopic metabolism of calcium in bone. IV. Ca^{45} deposition and growth rate in canine osteons. *Radiat. Res.* **10**, 243–257.

Marshall, J. H., Rowland, R. E. & Jowsey, J. (1959 b) Microscopic metabolism of calcium in bone. II. Quantitative autoradiography. *Radiat. Res.* **10**, 213–233.

Riggs, B. L., Kelly, P. J., Jowsy, Jenifer & Keating, F. R., Jr. (1965) Skeletal alterations in hyperparathyroidism: Determination of bone formation, resorption and morphologic changes by microradiography. *J. clin. Endocr.* **25**, 777–783.

Rowland, R. E. (1966) Exchangeable bone calcium. *Clin. Orthop.* **49**, 233–248.

Smeenk, D. (1961) Studies of bone of patients with hyperparathyroidism by means of phosphate exchange experiments *in vitro* and quantitative microradiography. *J. clin. Invest.* **40**, 433–444.

Stanbury, S. W. (1961) Bone biopsy in the study of metabolic bone disease. *J. Bone Jt Surg.* **43**-B, 602.

Strandh, J. & Bengtsson, A. (1961) The uptake of calcium in microscopic bone structures in compact bone. *Acta Soc. Med. upsalien.* **66**, 95–103.

Van der sluys Veer, J., Smeenk, D. & van der Heul, R. O. (1964) Tetracycline labelling of bone in hyperparathyroidism. In *Proceedings of the first European bone and tooth symposium, Oxford, April, 1963*, p. 85. Macmillan Co., New York.

Villaneuva, A. R., Ilnicki, L., Frost, H. M. & Arnstein, R. (1966) Measurement of the bone formation rate in a case of familial hypophosphatemic vitamin D-resistant rickets. *J. Lab. clin. Med.* **67**, 973–982.

APPENDIX

I. Skeletal sites studied in three representative dogs of different metabolic states.

A. Spinal column.

1. C-4.
2. L-2.
3. Second caudal vertebra

B. Ribs.

Right and left sides, third and eighth ribs (from each rib, sections were taken at the middle of the middle third and at the junction of the middle third with the anterior and posterior thirds).

C. Iliac crests.

1. Right and left sides, anterior iliac crest (1 cm behind anterior-superior iliac spine).
2. Right and left sides, posterior iliac crest (1 cm in front of posterior-superior spine).

D. Humerus, radius, ulna: right and left sides.

1. Diaphysis (midway between two epiphyses).
2. Metaphysis (proximal metaphysis in the humerus and distal metaphysis in the radius and ulna).

E. Tibia, femur: right and left sides.
   1. Diaphysis (midway between two epiphyses).
   2. Metaphysis (distal metaphysis in the femur and proximal metaphysis in the tibia).

F. Metatarsals: second and fourth (midshaft and anterior right foot only).

II. Skeletal sites studied in all dogs.

A. Iliac crest.
   1. Right and left sides; anterior iliac crest (1 cm behind anterior-superior iliac spine).
   2. Right and left sides; posterior iliac crest (1 cm in front of posterior-superior iliac spine).

B. Rib.
   Right and left sides, third and eighth ribs (from each rib, sections were taken at the middle third and at the junction of the middle third with the anterior and posterior thirds).

C. Tibia, femur: right and left sides.
   1. Diaphysis (midway between two epiphyses).
   2. Metaphysis (distal metaphysis in the femur and proximal metaphysis in the tibia).