Summary. The inhibitory action of heparin on prothrombin consumption of normal platelet-poor human plasma in a silicone-coated test tube can be partially overcome by increasing amounts of hemolysate. Likewise, prothrombin time of human plasma to which a fixed amount of heparin has been added can be shortened and brought to a constant but higher value than normal by adding increasing amounts of tissue thromboplastin.

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Quantitative Relationship of Osteoclasts to Parathyroid Function.* (25612)

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Problems concerning the role of osteoclasts in bone dissolution have long been investigated. Numerous studies have attempted to clarify this basic relationship, particularly in reference to the part played by osteoclasts in parathyroid hormone effect on bone resorption. These earlier studies have been reviewed by Hancox(1,2). Of particular interest are reports of Selye(3), which referred to increases in numbers of osteoclasts after parathyroid extract administration, and of Bloom *et al.*(4), which demonstrated qualitative increases in osteoclasts coupled with decreases in numbers of osteoblasts during laying cycle of pigeons. More recently Gaillard(5) noted significant increases both in number and activity of osteoclasts in incubated foetal bone, under the influence of a substance elaborated from concurrently incubated parathyroid tissue, or from parathyroid extract added to the media. Talmage *et al.*(6) were able to show qualitative increases in numbers of osteoclasts in long bones of rats undergoing continuous peritoneal lavage with calcium- and phosphate-free rinse. The only attempt to use quantitative determination of osteoclast concentrations as an index of metabolic activity has been the recent work by Myers *et al.*(7) on the mandibular condyle. The purpose of our study was to develop a method for quantitation of osteoclastic material which could be used as index of bone dissolution and of parathyroid activity.

Materials and methods. These experiments utilized approximately 75 male Holtzman rats with weight range restricted to between 225 and 250 g. All were placed on a calcium- and phosphate-free diet for 24 hr before start of each experiment. Bilateral nephrectomies and parathyroidectomies were performed under ether anesthesia. Continuous peritoneal lavage was carried out by introducing into the peritoneal cavity, through a stainless steel indwelling plug(8), 30 ml of a solution containing 0.8% NaCl and 0.5% glucose at pH 7.4. After equilibration for 1 hour, this fluid was removed and immediately replaced with another 30 ml of lavage solution. Animals were

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not anesthetized during lavage. Commercially available parathyroid extract,† when administered, was given intraperitoneally. *Histological procedures.* When animals were sacrificed, distal portion of the femur was exposed and the bone snapped at the epiphysis. Remainder of femur was sawed across the shaft one-fourth inch from the metaphysis. This distal segment, which included subepiphyseal and portions of the trabecular area, was fixed for 8 to 12 hr in Helly's fluid. After washing in water, bones were decalcified with several changes of 5% formic acid for several days. Following dehydration and infiltration with celloidin, they were embedded in paraffin and sectioned transversely at 7 μ. The area examined was that just beneath deepest penetration of epiphyseal plate into the diaphysis. This area represents, in all animals, the most uniformly growing area. Further, this technic permitted preparation of sections of each bone at exactly the same area of bone to insure maximum uniformity of conditions. Since quantitative counts of osteoclasts were to be made, a staining procedure was sought which would permit easy identification of these cells. After staining with Ehrlich's hematoxylin, slides were placed in a mixture of Biebrich scarlet and Orange II. Following treatment with phosphotungstic mordant, they were stained with Fast Green FCF, dehydrated and cleared. This procedure caused osteoclasts to appear as magenta colored cells in contrast to bone matrix which stained green and to erythrocytes which stained orange. For quick identification of osteoclasts this stain proved markedly superior to hematoxylin and phloxin tried earlier. *Counting technic.* By an ocular grid in binocular microscope at magnification of 430 ×, an area of bone 0.26 mm² was contained within the field of the grid. The entire cross-section of each bone was scanned and osteoclasts counted. Results were expressed as number of osteoclasts/field. Due to large size of an osteoclast, it was not routinely possible to determine which segments noted in the microscopic field made up a single osteoclast. Therefore, portions of osteoclasts which appeared as separate units in the field were counted separately. While this method does not give an absolute count of osteoclasts in a given area, it permits a quantitative representation of amount of osteoclastic material in a given volume of tissue.

† Eli Lilly Co., Parathyroid Extract.

TABLE I. Effects of Various Stimuli on Osteoclast Concentration in Rat Femurs.

	Group	No. rats	Osteoclasts/field*
1	Normal, control	32	3.55 ± .07
2	Normal, 250 USP PTH†	4	4.77 ± .44
3	PTX‡ 7 days	4	2.85 ± .18
4	PTX 7 days, 50 USP PTH	4	3.42 ± .49
5	NEPX§ 24 hr	16	6.95 ± .32
6	LAV¶ 12 "	9	4.54 ± .48
7	LAV 32 "	5	6.09 ± .18

* Field size 0.26 mm².
† Parathyroid extract given 18 hr before sacrifice.
‡ Parathyroidectomized.
§ Nephrectomized.
¶ Continuous peritoneal lavage.
Groups 2 and 3 are statistically different from Group 1 ($P = <.001$).

Results. In Table I are summarized results of a variety of experiments designed to show a quantitative relationship between parathyroid stimulation and osteoclast formation. The ratio found in animals maintained on stock diet served as the norm. Since 2 or 3 days on calcium- and phosphate-free diet did not significantly affect this norm, animals so treated were grouped with controls. Parathyroidectomy for 7 days reduced significantly the osteoclast count. Treatment of such animals with 50 USP units of parathyroid extract returned count to normal, as determined by bones removed 18 hr after hormone administration. Quantitative increases in osteoclast count above normal were produced by treatment with 250 USP units of extract, by technic of peritoneal lavage, and by nephrectomy. Degree of stimulation noted was maximum in bones taken from rats nephrectomized for 24 hr or from those undergoing continuous peritoneal lavage for 32 hr. Parathyroid extract (250 USP units) produced a stimulation in bones, removed 18 hr after administration, similar to that found in bones

removed after only 12 hr of continuous lavage.

A further study was made to determine if the ratio of cytoplasmic to nuclear material in these multinucleated cells varied with degrees of parathyroid stimulation studied. Counts of nuclei/osteoclast, however, remained constant even in bones showing a 100% variation in osteoclast count. The fact that number of nuclei/osteoclast remains constant, even though concentration of osteoclasts can change greatly, lends support to the idea that osteoclasts are formed by coalescence of other cell types; namely, osteoblasts and osteocytes.

Discussion. Our purpose was to determine if changes in parathyroid activity could be detected by quantitative change in osteoclast concentration of a selected area of long bone. Our data indicate such to be the case and also demonstrate that the procedure establishes a reliable index of changes in endogenous production of parathyroid hormone as well as those noted by exogenous administration.

The data also are of interest in regard to normal functioning of the parathyroid gland. According to work of McLean(9) and recent report of Talmage *et al.*(10), bone is under continuous influence of hormone from parathyroids due to necessity of "feeding back" calcium into body fluids. This concept is further substantiated by above data which indicate that normal osteoclast concentration can only be maintained in parathyroid-intact animals, and that a significantly lower level appears following parathyroidectomy.

A further point of interest is substantiation of hyperactivity of parathyroids following nephrectomy. This has been reported in studies of histological changes in the gland produced by nephrectomy(11,12). Degree of hyperactivity can be compared to animals lavaged continuously for 32 hr with a calcium- and phosphate-free rinse in which it can be calculated(13) that over 50 mg of calcium were removed from the animal. However, evidences of secretory changes in parathyroid tissue are most difficult to produce by means other than nephrectomy, and it is hoped that our technics will serve as a valuable aid in establishing physiological control of parathyroid secretion.

Summary. A method for quantitative determination of amount of osteoclast material in rat femurs is presented. The number of osteoclasts/unit area is sufficiently constant in normal animals to permit statistical differentiation between these and experimental groups. Parathyroidectomy reduced the osteoclast count, which was subsequently returned to normal by parathyroid extract administration. Nephrectomy, continuous peritoneal lavage, and parathyroid extract administration to normal animals, all increased significantly the number of osteoclasts/unit area. Maintenance of a normal osteoclast concentration above that of parathyroidectomized animals is discussed in relation to McLean's "feed-back" principle of parathyroid action under which continuous secretory activity of these glands is assumed. It is felt that determination of osteoclast concentrations as described can be used as index of parathyroid activity in studying problems related to parathyroid secretion.

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