A Radioautographic Study of Parathyroid Hormone and Calcitonin Binding *in vivo* to Various Tissues from Normal and Osteopetrotic Mutant Rodents

by

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Short title
PTH and CT binding in normal and mutant rodents

To
Richard, Marc-Antoine and Jessie-Jean

and
to everyone

who feels they have contributed to the realization of this work.

ABSTRACT

An in vivo competitive specific binding approach has been used to study the binding of parathyroid hormone and calcitonin in hepatic, skeletal, and renal tissues in normal rodents.

Salmon calcitonin (sCT) and an analogue of the bioactive NH_2-terminus of bovine parathyroid hormone, ([Nle-8,18,Tyr-34]bPTH(1-34)NH_2), were labeled with ^{125}I using the chloramine T method. Lactoperoxidase labeling was used to iodinate intact bovine parathyroid hormone bPTH(1-84).

Freshly radiolabeled, biologically active preparations were introduced intravenously **alone** or **mixed** with an excess of unlabeled related or unrelated hormone. After 2 minutes of circulation, animals were sacrificed by intracardiac perfusion. Tissues were removed and prepared for light, and electron microscope radioautography, after glutaraldehyde fixation.

Competitive, specific binding for bPTH was found over the cell surface of hepatocytes, osteoblasts, and primary foot processes of renal podocytes. Binding was also related to the antiluminal aspect of cells forming the wall of the proximal tubular portion of the nephron and related to some of the cellular elements in the walls of the distal portion of the nephron. Calcitonin binding was observed related to hepatocytes, osteoclasts, and to the antiluminal aspect of cells forming the walls of the distal portion of the nephron.

The qualitative analysis of the binding at the electron microscopic level using the in vivo approach suggests the presence of three classes of receptors, based on the ability of related unlabeled hormone to compete either maximally, to a lesser extent, or apparently not at all with labeled hormone for receptor binding.

The method developed to examine normal animals was then applied to examine CT binding in osteopetrosis, a disease characterized by abnormal bone resorption and remodeling. Binding in the osteopetrotic mutants was compared with that in their phenotypically normal siblings. A statistically significant increase in cell surface binding over osteoclasts, in osteopetrotic op/op mice and in microphthalmic mi/mi mice, was demonstrated. A virtual absence of binding, in osteosclerotic oc/oc and incisor absent ia/ia mutant rodents, was found.

In addition to the skeletal abnormalities, alterations in liver and kidney binding, as well as ultrastructural abnormalities were observed in some of these mutants suggesting extra-osseous disease in these animals.

RESUME

Une méthode in vivo de liaison compétitive a été utilisée pour étudier la liaison de l'hormone parathyroidienne et de la calitonine au tissues hépatique, osseux et rénal chez des rongeurs normaux.

La calcitonine de saumon (sCT) et la partie NH_2-terminale bioactive de l'hormone parathyroidienne ont été marquées avec l'iode 125 en utilisant la méthode à Chloramine T. La méthode à la lactoperoxidase a été utilisée pour l'hormone parathyroidienne (bPTH).

Chaque hormone fraîchement marquée et biologiquement active a été injectée i.v. en absence ou en présence d'un excèss d'hormone froide, apparentée ou non. Deux minutes après, les animaux ont été sacrifiés par perfusion intracardiaque. Les tissues ont été prélevés, fixés au glutéraldéyde et préparés pour la radioautographie à la microscopie optique et électronique.

Une liaison spécifique de la bPTH a été observée à la surface des hépatocytes, des ostéoblastes et des extensions cytoplasmiques des podocytes. La liaison a été aussi observée sur la surface basale des cellules formant le mur de la portion contournée des tubules proximaux du néphron et sur la surface basale des cellules formant le mur de la portion distale du néphron. La liaison spécifique de la sCT a été observée sur les hépatocytes, les ostéoclasts et la surface basale des cellules formant le mur de la portion distale du néphron.

L'analyse de la liaison suggère l'existence de trois classes de récepteurs, basée sur la capacité de l'hormone froide à entrer en competition avec l'hormone marquée au niveau du récepteur.

Cette méthode a été utilisée pour étudier la liaison de la sCT chez des rongeurs ostéopétrotiques et la reaction a été comparée a celle obtenue dans les animaux phénotypiquement normaux de même portée. Une augmentation significative de la liaison à la surface cellulaire des ostéoclasts a été démontrée chez les souris ostéopétrotiques op/op et microphthalmiques mi/mi. L'absence de liaison chez les souris ostéosclérotiques oc/oc et les rats sans incisive ia/ia a été observée.

En plus des anomalies au niveau osseux, des altérations de la liaison de la sCT, ainsi que des anomalies ultrastructurelles au foie et au rein ont été relevées chez certain de ces mutants, suggérant des atteintes extra-osseuses chez ces animaux.

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PART-I

LIST OF ABBREVIATIONS

ACT	Arched collecting tubule
ACTH	Adrenocorticotrophic hormone
B	Bone
BB	Brush border
bc	bile canaliculus
bPTH	Bovine parathyroid hormone
bv	Blood vessel
C	Cartilage
CAL	Cortical thick ascending limb
cb	cytoplasmic body
CCT_b	bright portion of the cortical collecting tubule
CCT_g	granular portion of the cortical collecting tubule
ct	connective tissue
cmp	counts per minute
CNT	Connecting tubule
CT	Calcitonin
cz	clear zone
DCT	Distal convoluted tubule
DCT_b	bright portion of the distal convoluted tubule
DCT_g	granular portion of the distal convoluted tubule
E	Endothelial cell
EM	Electron microscope
fp	podocyte foot process
FSH	Follicle stimulating hormone
G	Golgi
GAG	Glycosaminoglycans
gr	gram
H	Hepatocyte
H	Hormone
K	Kupffer cell
LC	Liver cord
LH	Lutenizing hormone
LM	Light microscope
M	Mesangial cell
MAL I_s	Medullary thick ascending limb, inner stripe
MAL O_s	Medullary thick ascending limb, outer stripe
m	mitochondria
ml	milliliter
mv	macropinocytotic vacuole
mm	millimeter
ms	mixed spicule
N	Nucleus
n	nucleolus
OAF	Osteoclast activating factor
ob	osteoblast
ostc	osteoclast
PT	Proximal tubule
PCT	Proximal convoluted tubule
PR	Pars recta

PTE	Parathyroid Extract
PTH	Parathyroid hormone
PTS_1	Proximal tubule, segment 1
PTS_2	Proximal tubule, segment 2
PTS_3	Proximal tubule, segment 3
R	Receptor
RC	Renal corpuscle
RB	Ruffled border
S	sinusoidal cells
s	sinusoid
sCT	salmon calcitonin
µg	microgram
µl	microliter
µm	micrometer
v	vesicle
va	vacuoles

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PREFACE

This thesis is a qualitative and a quantitative analysis of the *in vivo* binding of parathyroid hormone and calcitonin in the hepatic, skeletal, and renal tissues of normal and osteopetrotic mutant rodents. There are four major parts to the thesis.

In the *first part* the two hormones under study are introduced and the notion of a receptor, as well as the various approaches used to study the hormone-receptor interaction are presented. It continues with an introduction on osteopetrosis and a brief description of the four mutants investigated.

In the *second part* the *in vivo* competitive specific binding approach is presented together with the technique used for iodination of the hormones. The animal procedures, consisting of the intravenous injection and whole body perfusion, as well as the processing of the tissue samples for light and electron microscope radioautography is described. The rigid criteria used to quantitate the radioautographic reactions are included.

In the *third part*, the competitive specific and the non-competitive binding for ^{125}I-bPTH and ^{125}I-sCT experiments at the light and electron microscopic level in normal and osteopetrotic mutant rodents, are described. The statistical significance of differences observed between reactions in animals injected with radioactive hormone **alone**, and **co-injected** with excess related or unrelated unlabeled hormone, are presented.

In the *fourth part*, results of the *in vivo* competitive and non-competitive binding studies for bPTH and sCT in hepatic, skeletal, and renal tissues are correlated with knowledge of hormonal sites of action and physiology.

The existence of three classes of receptors is postulated based on the results of competition studies for receptor occupancy using homologous unlabeled hormone.

Finally, alterations in calcitonin binding to osteoclasts of osteopetrotic mutant rodents are discussed in terms of receptor modification or regulation. The biological significance of the abnormal kidney structure, which was found in these mutants, is discussed.

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PART I

INTRODUCTION

The prerequisites to study alteration in hormone-target cell interactions are the identification of the cells bearing the specific receptors for a given hormone, as well as the acquisition of some knowledge on the topography and density of receptors in normal animals.

Although much scientific literature already describes PTH and CT involvement in osteogenesis and osteolysis, there is still a great deal of uncertainty about which cells are acted upon by which hormones, as well as on the exact role which these hormones play in the modulation of these processes.

Taking advantage of the in vivo competitive specific binding approach, the nature of the target cells for PTH and CT has been determined. The topography, the density, and the pattern of cellular uptake has been studied in hepatic, skeletal, and renal tissues in normal rodents.

Using the same approach, sCT binding was studied in a variety of osteopetrotic mutants. Osteopetrosis is a skeletal disease characterized by an increase in bone density and abnormal bone resorption due to defective osteoclasts (see review in Marks and Walker, '76). The models studied include the osteopetrotic op/op, microphthalmic mi/mi, and the osteosclerotic oc/oc mice, as well as the incisor absent ia/ia rat. Data obtained from the osteopetrotic mutants has been compared to that obtained from their phenotypically normal siblings.

Calcium Regulating Hormones

A quarter of a century ago, theories of hormone action were neglected or rarely considered. Endocrinologists were concerned with hormone physiology and resulting bio-responses. This interest was shared by the biochemists who focused their attention on the isolation, purification, characterization, and biosynthesis of hormones.

Hormones are chemical compounds which are synthesized and released by a body of cells, collectively referred to as the glandular tissue. They are known to travel in the blood circulation to distant groups of cells, referred to as the target tissues, although the released factors may also act on neighbouring cells. Many circulating or in situ factors have been identified as hormones, and every day, more are added to the list. They are generally divided in two groups on the basis of their solubility : the lipid soluble, among which are the steroids, and the water soluble, the polypeptide hormones which constitute the majority of known hormones. Parathyroid hormone and calcitonin, the two hormones involved in this study, belong to the water soluble group.

Calcium homeostasis in extracellular fluid involves the complex interactions of both peptide and steroid hormones with several tissues (Cole et al., '81). Parathyroid hormone and calcitonin have long been considered for their involvement in calcium regulation.

Parathyroid hormone

Molecular structure

Parathyroid hormone (PTH) was first extracted from bovine parathyroid glands by Collip in 1925. Structurally, it was subsequently shown to be a single-chain polypeptide of 84 amino acids with a molecular weight of 9500 (see review in Martin et al., '79). There are no cysteine residues and hence, no disulfide bridges. There is a preponderance of basic residues (arginine and lysine), conferring an overall positive charge to the molecule (Keutmann, '79).

In the circulation, the hormone exists in more than one immunoreactive form. The heterogeneity of the bioactive forms (Goltzman et al., '84) is not as pronounced as that of the immunoreactive forms (Berson and Yalow, '68). Although the fragments found in the plasma can be secreted by the parathyroid glands (Flueck et al, '76), there is strong evidence that the peripheral organs contribute to the heterogeneity of the circulating forms. PTH fragments appear in the circulation after a single injection or by a constant infusion of labeled or unlabeled intact bovine PTH (Habener et al., '71). Cleavage occurs in the middle portion of the intact PTH, and results in an apparent biologically active amino-terminal fragment (NH_2-terminal), the fate of which is unclear at the present time, and a biologically inactive carboxy-terminal fragment (COOH-terminal) (see review in Martin et al., '79).

The minimum sequence requirement for biological activity includes a continuous sequence from residue 2 extending to, at least, residue 27 of the molecule (Tregear et al., '73). The methionines in position 8

and 18 appear to play a major role in the expression of biological activity (Goltzman et al., '75). Intact hormone can stimulate adenyl cyclase activity in vitro in renal cortical membrane (Goltzman et al., '76) and rabbit calvarial membrane (Goltzman, '78), indicating that metabolism of the hormone in these two main fragments, is not a necessary step for binding and adenyl cyclase activation in known target tissues.

Secretion

The extracellular fluid calcium level is the major factor altering the release of the hormone from the parathyroid glands (Sherwood et al., '68). The observation of 1, 25-dihydroxyvitamin D-binding proteins within parathyroid cells suggests that vitamin D might exert some feedback control over the release of the peptide hormone (Chertow et al., '75).

Site of action and physiology

Physiologically, parathyroid hormone is the most important regulator of extracellular calcium concentration. Calcium homeostasis is the result of the direct action of PTH on bone and kidney, and an indirect action on the gastro-intestinal tract.

PTH is known mostly for its catabolic action on the skeleton (Talmage, '67). The increase in bone resorption is only part of a complex bone response which apparently affects all of the bone cell types and involves not only catabolic effect but also anabolic effects (Pugsley and Selye, '33). Some have suggested that at least at physiological concentration, it is the anabolic function that predominates (Parsons and Reit, '74).

The effects of PTH on the kidney are multiple. In the proximal tubule portion of the nephron, PTH influences phosphate, bicarbonate

and sodium transport (Agus et al., '73). Calcium transport is enhanced by PTH action on the distal portion of the nephron (Windrow and Levinsky,'62)

In the kidney, PTH enhances the production of 1,25 dihydroxycholecalciferol (DeLuca, '73), and stimulates mammalian renal 25-hydroxyvitamin D_3-1 alpha-hydroxylase in vitro (Kremer and Goltzman, '82). The vitamin D metabolite then stimulates calcium and phosphate absorption in the intestine (Nicolaysen et al., '53), and together with parathyroid hormone bring about normal bone mineral mobilization (see review in DeLuca and Schnoes, '76).

Calcitonin

Molecular Structure

Calcitonin (Copp and Cameron, '61), previously named thyrocalcitonin (Hirsch et al., '63), is a polypeptide hormone first identified in rats (Copp et al., '62). It has been purified and sequenced from a number of species including man, cow, pig, goat, chicken, salmon, and eel (see review in Hirsch and Munson, '69).

The molecular structure consists of a single peptide chain of 32 amino acids in length with a molecular weight of approximately 3,300 daltons. There is a disulfide bridge between position 1-7 at the amino terminus and a proline amide at the carboxyl end (Potts and Aurbach, '76).

The retention of the disulfide bridge, as well as that of the carboxy terminal proline amide, appears essential for the preservation of biological activity (Rittel et al., '76). There is variation in amino acid sequence between various species which is difficult to correlate with different biological potency. Among the most potent forms are

those derived from the ultimobranchial bodies such as salmon calcitonin.

Secretion

The hormone is synthetized by the clear cells found in parafollicular position in the mammalian thyroid gland (Foster et al., '64), and in the ultimobranchial bodies in submammalian species. The rate of secretion increases in direct relationship to the degree of hypocalcemia (Deftos et al., '68). Its release can be induced by superphysiological levels of glucagon and by perfusion of the thyroid gland with dibutyryl cyclic AMP, as well as by gastrin. It can be augmented by calcium, magnesium, and glucagon. It is also increased by thyroxine, pancreozymin, gastrin, pentapeptide, acetylcholine, epinephrine, prostaglandins, serotonin, histamine, and is inhibited by norepinephrine (Bell, '70). Moreover, calcitonin plasma level and secretion varies with species, sex, age, nutritional, and reproductive status of the animals (Munson, '76; Toverud and Boass, '79; Birge et al., '81).

Site of Action and Physiology

Zondec and Ucko in 1925, first reported calcitonin's calcium lowering ability. They reported that thyroid extract lowered serum calcium in rabbits by 30% (see review in Hirsch and Munson, '69). Its role as a hypocalcemic factor was firmly established by the observation that in rats, the calcium level, after injection of calcium-chloride was much higher in the absence of the thyroid gland than in its presence (Talmage et al., '64).

Neither nephrectomy or removal of the gastro-intestinal tract prevented hypocalcemia in rats after administration of sCT (Munson et al., '66) suggesting that osseous tissue is the site where

the characteristic hypocalcemic effects were produced. Calcitonin inhibits PTH-induced release of ^{45}Ca from pre-labeled bone in tisue culture (Freidman and Raisz, '65). Circulating, as well as urine levels, of ^{3}H-hydroxyproline (Aer, '68), together with a reduction in osteoclast number (Reynolds et al., '68), and changes in the ultrastructure of these cells in CT-treated system suggested a reduction in osteoclast activity (Holtrop et al., '74). **In vivo** calcitonin treatment decrease acid phosphatase production, and the enzyme is not secreted at the bone-cell interface (Lucht, '73).

Because the presence of the thyroid gland is not essential for the maintenance of normal calcium, a main role for calcitonin in its homeostasis seems unlikely. A possible role in phosphate homeostasis has been proposed. While calcitonin inhibits the entrance of calcium into the plasma, it also increases the rate at which phosphate leaves the plasma. The plasma phosphate falls following hormone injection and this fall is often greater than that of calcium and can be produced in the absence of any hypocalcemic effect. Calcitonin certainly protects against hypercalcemia produced artificially by injection of calcium salts, or that occurring post prandially, it may serve additionally to protect the skeleton against excessive resorption by PTH, especially during the periods of calcium deprivation, or increased calcium demands.

Effects on the gastro-intestinal tract include secretion of water into the jejunum as well as sodium, chloride, and potassium into the intestinal lumen (Gray et al., '76). In man and in all animal species so ar investigated, injection, infusion, or oral administration of calcitonin produces a dose-dependent lowering of the volume of secretion of

free or total acid, and of the pepsin secretion in the stomach (Doepfner and Briner, '80; Hotz et al., '80), actions that may be mediated through the subfornical organ of the brain (Rouleau et al., '84).

Studies of Hormone-Receptor Interaction

Receptor

Concepts and definitions

The receptor concept can be traced to the early 1900's when Langly, Clark, and Ehrlich, investigating the action of tetanus toxin, concluded that the toxin had to unite with some receptive substance in order for it to have its effect (see review in Vallentine and Hollenberg, '84).

The first definition of a receptor, dictated by the analyis of bio-responses, was endowed with the dual functions of hormone recognition and cellular activation. As of today, in pharmacological terms, the membrane receptors are defined as macromolecules that recognize a ligand of interest in a chemically specific manner which causes an immediate perturbation of the membrane function that in some manner leads to the biological responses.

In the scientific literature, the term receptor is currently used to describe this cellular component which recognizes a specific ligand and sets in motion a sequence of events which culminates in a characteristic bio-response. Most writers still do not distinguish the recognition site, that is the binding site, from the whole multimolecular complex.

Hence, depending on the type of approach and discipline used to investigate its nature, whereby only the recognition event, or the recognition and activation event are observed, the term receptor may be used in a somewhat broader, or more restrictive sense by emphasizing one aspect of the receptor over another.

Structures

From biochemical studies, it appears that a receptor consists of a set of related molecular species present in a single membrane system (Hecthter and Braun, '72). There is enormous variability in the size, the chemistry, the three-dimensional arrangement as well as in the functions of the constituting units.

The most extensively characterized receptors are those for gonadotropins, acethylcholine, insulin, and epidermal growth factor. Glucagon and prolactin receptors have been isolated, purified, and studied as well. Generally speaking, polypeptide hormone receptors are glycoproteins in nature. They have an estimated molecular weight in the order of 200,000 to 500,000 daltons which exceeds by far the molecular weight of the hormones they bind. As many as five different subunits have been described for the acetylcholine receptor, two of which are believed to be involved in the hormone recognition function, the three others may participate in the formation of an ion channel (Meunier et al., '72; De Meyts, '76). Viewed with the electron microscope, the purified receptors appear as ring-like particles of 8-9 nm in diameter surrounding an electron dense pit (Cohen and Changeux, '75).

In many cases, including calcitonin and parathyroid hormone, the generation of the bio-response involves the initial activation of the

adenylate cyclase system. Hence, in these cases the receptor may be viewed as a recognition unit not associated with adenylate cyclase the catalytic unit, their association requiring the participation of an additional component the regulatory protein unit (N), a nucleotide which acts as an intermediate between the receptor and the catalytic unit.

Location

Evidence indicates that for polypeptide hormones, which are water soluble factors, the initial site of interaction is on the cell surface. Polypeptide hormones attached covalently to insoluble polymers such as agarose can form strong complexes on cell surface situated structures. Trypsin, insoluble trypsin, and chymotrypsin-derivatives destroy cell responsiveness by modifying some important cell surface elements (Lefkowitz et al., '71; Cuatrecasas, '71).

Highly purified plasma membrane subcellular fractions are greatly enriched with receptors and labeled hormone binds to one face only of these membranes. This indicated that the receptor molecules were integrated in the membrane in a polar manner so as to have the recognition site exposed to the extracellular milieu (Jarret and Smith, '74). Direct evidence for the localization of insulin receptors has been obtained from extensive binding studies that utilize highly radioactive ^{125}I-insulin and isolated cells or microsomal preparations (Cuatrecasas, '74). At least in some cases, they are known to form patches, or clusters. The phenomena referred to as **capping** has been demonstrated for concanavaline A, immunoglobulins, and antigen on lymphocyte surface indicating that the receptors are not fixed elements in the fluid mosaic plasma membrane (de Petris, '75). Their number and affinity can be regulated by intra- and extracellular mechanisms.

Receptor Regulation

It is clear that, in order to act, a hormone must first establish contact with the receptor. The recognition event is followed by cellular activation so as to generate the appropriate bio-response. From mathematical relationships, it has been proposed that the cellular response depends on the number of hormone-receptor complexes present (Cuatrecasas and Hollenberg, '76). In many tissues, the complete biological response is evoked by occupancy of a small proportion of the available receptors. The presence of a larger number of receptors, **spare receptors**, could be essential to provide for occupancy of the much smaller number required for biological response (Stephenson, '56). Although it is still not clear whether cellular sensitivity is mostly related to the number of receptors per cells, per square micrometer, per receptor cluster, per milligram membrane protein, or per unit of adenyl cyclase and 5' nucleotidase activity, at the present time, hormone sensitivity seems to correlate best with the number of receptors per unit surface area.

It has become evident that not only does hormone concentration fluctuates widely in response to changes within an organism, but the concentrati and affinity of receptors for this hormone also changes rapidly in response to extracellular as well as intracellular signals (Williams, '81).

Genetic regulation of the receptors

The type and the number of receptors on cells are highly dependent on the cell's genetic make-up and developmental program. Normal events such as differentiation and growth are often associated with major changes at the level of the receptors.

Rapidly growing cells exhibit less receptors for insulin than

mature cells. There is a marked increase in concentration of insulin receptors on fibroblasts as the cell enters a confluent stationary growth phase (Thomopoulos, see review in Kahn, '76).

Modulation of the synthesis and internalization

The concentration of hormone receptors, like that of other membrane components, is not fixed. It is a reflection of a state of continual synthesis and degradation that is subject to regulation by intracellular as well as extracellular factors. Not much is known about the normal rate of receptor turnover. Insulin receptors are lost at the rate of 20% per hour, and apparently 50% of the receptors are renewed within 40 hours.

Self regulation of membrane receptors is a common phenomena. The exact mechanism by which it occurs is uncertain. **Down-regulation** as well as **up-regulation** have been documented. Binding of ligand may promote degradation of receptors by either membrane associated protease, endocytosis, or shedding of the hormone receptor complex into the medium (see review in Kahn, '76). Prolactin receptors are under positive feedback regulatory control by prolactin (Posner et al., '74).

Hormonal regulation of receptors

The receptor may be regulated by the hormone it binds specifically (homospecific regulation), or regulated via another hormone (heterospecific regulation) (Roth et al., '79).

Homospecific regulation of receptors. A decrease in the concentration of homologous hormone is followed by an increase in the concentration of receptors, a phenomena referred to as **up-regulation.** An example of this type of regulation is that of insulin deficient states where there is

an increase in receptor concentration on the cell membrane (Kahn et al., '73; Soll et al., '75). Parathyroidectomy, which decreases serum calcitonin concentration, is associated with increased calcitonin binding to the membrane of rat kidney (Sraer et al., '74).

In the case of excess hormone, as in the hyperinsulinemic, and resistant obese hyperglycemic mouse, an increase in the concentration of homologous hormone, is followed by a decrease in receptor number and is referred to as **down-regulation.** Extensive study of fully purified plasma membrane fractions, obtained from the obese hyperglycemic mice, has shown a 70% decrease in insulin receptors but they remain identical to those of their normal littermate with respect to 5'-nucleotidase adenyl cyclase activity, protein subunit composition, affinity, kinetic of association and dissociation, temperature dependence of binding and biological specificity (Soll et al., '75).

The cellular processes by which homologous hormones regulate their own receptors appear to be multiple. These include reverse inactivation at the cell surface, a modulation of the biosynthesis or membrane incorporation of the newly synthesized receptors, and a modulation of receptor internalization, a phenomena which may or may not be accompanied by accelerated destruction.

Heterospecific regulation of receptors. One hormone may affect the interaction of another, this process can be either direct, by affecting the target cell, or indirect, by affecting the function of other cells that ultimatly alter the target cells. Heterologous effects appear to be widespread and are part of a general phenomena called **priming**, where the action of a hormone is a prerequisite for the development of the

sensitivity of a target cell to another hormone. Follicle stimulating hormone (FSH) administration to animals results in an increase in the number of LH receptors in the ovary (Zeleznik et al., '74).

Effects of physico-chemical environmental factors

In the in vitro approach, structure and function of the receptors and hormones as proteins are heavily influence by physico-chemical environmental factors such the solvent, the ionic composition including the pH, and the temperature. The binding of insulin is pH and temperature dependent. ACTH binding to its receptor has a strong inverse relationship with the Ca^{++} concentration. Angiotensin binding is related to the concentration of Na^{+}. It seems logical that this phenomena can be extended in vivo, that is, changes in extracellular fluids may influence the receptor expression or its binding.

In addition to direct effect on the proteins, the physico-chemical environment can affect the lipid matrix of the plasma membrane and thereby affect indirectly the structure and function of the integral protein of the cell membrane. It has been reported that the type and the proportion of lipid components of the membrane strongly influences the function of the receptor and other integral membrane proteins such as adenylate cyclase (Williams, '81). Membrane lipids are rapidly exchangeable with the lipids of the environment. Consequently, changes in plasma lipids can result in changes in membrane lipids, which in turn may affect the behavior of the membrane proteins.

Changes in affinity

A much less common mechanism by which a hormone may regulate the sensitivity of its target cell is by affecting receptor affinity. In

the case of insulin, progressive saturation of receptors progressively lowers the affinity of all of the receptors for that hormone, this is due largely to an instantaneous increase in the rate of dissociation of hormone from the receptor which results in a decrease in the mathematical estimation of the affinity constant.

$$K_A = K_{ass} / K_{diss} \qquad (4)$$

The cooperativity principle implies site-site interaction, or cooperativity among the receptors. The reaction is considered **positive cooperativity** if the binding of the first ligand increases the affinity of the receptor for a second ligand as is the case for hemoglobin, where binding of one oxygen enhances the affinity of both oxygen-binding sites. In this case the phenomenon is referred to as positive cooperativity. It is **negative cooperativity**, if the binding of the first ligand decreases the affinity of the receptor for the second (de Meyts, '76). **Heterotropic cooperativity** occurs when the affinity of a hormone receptor is altered by the binding of a different ligand to the receptor. Divalent cation concentration, and pH, also have profound effects on binding of some hormones, suggesting that these two may act as heterotropic regulators of receptor affinity (Rodbell et al., '71). The possible mechanism to explain these various cooperative effects includes, (1) changes in the oligomeric receptors, (2) polymerization (clustering) or depolymerization of receptor in the fluid membrane.

Mathematical considerations on receptor regulation

The hormone-receptor interaction can be represented as a dose-response curve of bio-effect versus hormone concentration. At fixed hormone concentrations, an increase in receptor concentration shifts the dose-response curve to the left, indicating an increase in the effectiveness for a given concentration of hormone to generate a bio-response. A decrease in receptors shifts the dose response curve to the right indicating a decrease in effectiveness for a given concentration of hormone to bring about the bio-response (Williams, '81).

Since the affinity is a mathematical function of [H] [R] and [HR], in the mathematical relationship, $k_{ass}/k_{diss} = [H][R] = K_A$, a change in receptor concentration, mathematically speaking, results in a change in affinity constant K_A. Moreover, affinity is a function of the association and dissociation rate constant. At fixed hormone concentration, and at fixed receptor concentration, given that the alteration is in the chemical interaction between the hormone and the receptor which may be genetically or cellularly regulated, a change in affinity in essence brings about changes in the kinetics of H and R association and dissociation, and consequently changes in the mathematical expression of the affinity.

An **increase in affinity** typically slows the dissociation rate until at some limit, no dissociation occurs. **A decrease in affinity** suggests a slower rate of association, if any association at all. Because essentially the chemical bond provides selective recognition (specificity), and determines the strength of the linkage (affinity), extreme changes in affinity may not be truly independent of specificity.

Hormone-receptor interaction

Indirect approach

Knowledge about hormone-receptor interaction was first obtained from analysis of bio-response curves generally involving the injection of large amounts of hormone. The analysis of the dose-response relationships was achieved in a way similar to the evaluation of substrate-velocity relationships for enzymes. In that system, the final assumption that **binding** was **receptor binding** relied mainly on the correlation between the introduction of hormonal agents to the system and the generation of bio-responses.

The indirect approach of the classical pharmacologist to study the hormone-receptor interaction has helped delineate structural, kinetic, and physiological criteria that characterized a number of hormone-receptor interactions. Moreover, it has been used as the basis for evaluating the more direct analysis of ligand-receptor interactions. Their approach consisted in the use of agonist probes (active hormones or analogues which have an affinity greater than zero in a given system), or antagonist probes (substances which have an affinity greater than zero with an intrinsic activity equal to zero).

Measurements of hormone-dependent adenyl cyclase activity, or the production of the so-called second messenger cAMP serves as a means of establishing whether or not receptors for a given hormone are present in a biological system (Sutherland et al., '68).

Direct in vitro approach

Technical innovations, in particular the development of high specific

activity radiolabeled ligands, and conceptual innovations as in the principles first formulated in studies of polypeptide hormone-specific antibody interaction, have contributed greatly to establishing the basis for the direct approach for studying hormone-receptor interactions. In fact the entire concept employed in the radioimmunoassay field were appropriated for current receptor studies (Yalow and Berson, '60).

Several review articles discuss the in vitro approaches used to study the hormone-receptor interaction (Roth, '73; Cuatrecasas, '74; Cuatrecasas and Hollenberg, '76; Kahn, '76; Vallentine and Hollenberg, '84). The most widely used direct in vitro approach, applicable for all polypeptide hormone interaction, was introduced in the late 60's for the study of ^{125}I-ACTH and ^{125}I-angiotensin (Lefkowitz et al., '69; Goodfriend and Lin, '69; Braun et al., '70).

This method basically utilizes the in vitro interaction of receptors, radiolabeled hormone, and unlabeled hormone. The underlying principle is that of competition between labeled and unlabeled hormone for receptor occupancy. Labeled hormone, bound to receptors, can be progressively displaced by increasing concentration of unlabeled homologous hormone for occupancy of the limited number of receptor sites.

The receptors may be in organ or cell cultures, or in tissue or cellular homogenates. In broken cell preparations, the receptors are localized, especially, but not exclusively, to the plasma membrane subcellular fraction. They may be solubilized in detergents or in aqueous solutions.

In the in vitro approach, the hormone may be tagged with fluorescent, radioactive, or electron dense moieties. The marked ligands have been tracked to their site of interaction using ultra-

sensitive photo-receptor, light or electron microscopy, or radioautography depending on the nature of the marker. The hormone and the receptors are allowed to interact for an appropriate amount of time; afterwards, unbound hormone is separated from receptor-bound hormone, an important step before the estimation of activity retention by the system. When the receptors are on isolated cells, or in subcellular fractions, centrifugation or filtration on millipore filters are the most commonly used methods. When the receptors are soluble, gel filtration can be used. Quick methods for separation include the use of talc and silica, which absorb free hormone, or the use of polyethylene glycol, which precipitates bound hormone.

When the ligand is radiolabeled, the radioactivity content, an indicator of binding, is determined with a radiation gamma scintillation counter, and the data expressed in dose-response graphs. In this case, **total binding** is the radioactivity retained by the membrane or cell pellets. **Non-specific binding** is that portion of the total binding which remains in the presence of excess unlabeled related hormone, and represents an unsaturable component frequently called non-displacable. **Specific binding** is a mathematically derived measure obtained by subtracting the non-specific from the total binding. It is saturable and presumably receptor-bound.

If the membrane or cell preparations are processed for radioautography, the radioactivity is visualized as silver grains in the photographic emulsion. **Total binding** is the number of silver grains in radioautographs prepared from systems treated with the radiolabeled material exclusively. **Non-specific binding** is the radioactivity not - displaced by excess unlabeled related hormone. **Specific binding**

is the radioactivity displaced by unlabeled related hormone (Junger, '78).

A variety of mathematical methods have been applied to analyse the steady state binding data of the classic dose-response curves. Overviews of the methods, their assumptions and limitations, have been reviewed (Rodbard, '73; Kahn, '75; Birnbaumer et al., '74; de Meyts, '76).

In most of these analyses, the hormone interaction has been represented as a simple reversible chemical bimolecular equilibrium. This relatively simple equilibrium forms the basis of a number of drug and hormone theories and may be represented as in equation (1).

$$[H] + [R] \underset{k_{diss}}{\overset{k_{ass}}{\rightleftharpoons}} [HR] \qquad (1)$$

Where, **[H]** is the concentration of free hormone

[R] is the concentration of unoccupied receptors

[HR] is the concentration of hormone-receptor complexes

k_{ass} and k_{diss} are the association and the dissociation rate constants

From the analysis of dose-response curves, various hormone-receptor interaction characteristics and properties have been determined (Roth, '73; Cuatrecasas, '74). Receptor binding is a function of hormone [H] and receptor [R] concentrations. The ability of a given hormone receptor preparation to reach a plateau in the presence of increasing concentration of labeled hormone is referred as saturability. It indicates that the amount of binding sites is finite and, at the plateau concentration, all receptors are occupied. The interaction is pH, time, and temperature dependent. At 37°C, with high physiological

hormone concentrations, steady-state is achieved in a few minutes. At low temperature, and low hormone concentrations, a steady state may not be reached for hours. The hormone receptor interaction is reversible. At equilibrium, receptor bound hormone can be progressively replaced by excess unbound related hormone. Other binding properties, including affinity and specificity, have been determined as well.

Binding, in essence, is the result of non-covalent chemical interactions between the hormone and the recognition site of the receptor. It involves electrostatic interactions including hydrogen bonds, hydrophobic interactions, and Van der Walls forces (Baxter and Funder, '79).

Attempts have been made to mathematically characterize the affinity for a variety of hormone-receptor interactions. Equation (1) represents the simple bimolecular representation of the hormone-receptor interaction. At equilibrium, since the association rate constant k_{ass}, and the dissociation rate constant k_{diss}, are fixed, the rate of association is equal to the rate of dissociation.

$$k_{ass}\,[H]\,[R] = k_{diss}\,[HR] \qquad (2)$$

$$k_{ass}/k_{diss} = [HR] / [H]\,[R] = K_A \qquad (3)$$

The relationship k_{ass}/k_{diss}, at equilibrium, is the **equilibrium association constant,** also represented as the K_A. For a substance which is not active in a system, K = 0. For a substance which is active in the system, $K > 0$.

By simple mathematical manipulation, equation (2) may also be expressed as

$$k_{diss}/k_{ass} = [H]\,[R] / [HR] = K_D \qquad (4)$$

where K_D, the affinity, may also be expressed as the **dissociation equilibrium constant.**

In both equation (2) and equation (3), when [R] and [HR] are equal, that is, at half saturation of the receptors, free receptors [R] and hormone-bound receptors [HR] are equal, consequently the mathematical relationship (2) becomes

$$K_A = 1 / [H] \quad (5)$$

relationship (3) becomes

$$K_D = [H] \quad (6)$$

and by substituting (equation 6) in (equation 5),

$$K_A = 1 / K_D \quad (7)$$

Thus the free concentration of hormone necessary to saturate half the receptors becomes an important variable in the mathematical estimation of the affinity. It has been observed that for most peptide hormones, the K_A is remarkably high, 10^8-10^{10} M^{-1}, with K_D less than 10^{-8} M. These receptors are low capacity sites, in some cases with less than 10^5 receptors per cell.

Specificity is the ability of a receptor to recognize a bioactive hormone from among all other substances to which it is exposed. Hence, specificity is demonstrated when binding of the radioactive hormone is blocked by the co-injection of homologous unlabeled hormone, but not by co-injection of heterologous, structurally dissimilar unlabeled hormone.

Direct in vivo approach

Experiments done in vivo, in an undisturbed organism, are designed to follow the fate of injected radiolabeled hormone under normal physiological conditions. In the past, the low specific activity of the labeled preparations, and the low biological activity,

made this method unpopular. Although it was of some use in establishing total binding, it could not be used to distinguish between hormone receptor binding versus other binding. Hormone-receptor binding was dwarfed by the presence of intact and degraded radioactive hormone free in the adjacent extracellular fluid or adsorbed to relatively non-specific sites on cell surfaces (Roth, '73).

Major improvements were brought about with the use of high specific activity radiolabeled ligands and high biological activity. The introduction of perfusion with Ringers' solution to remove unbound circulating ligand, and the use of control animals, injected with excess unlabeled related hormone, facilitated the use of the in vivo method in a way anologous to the competitive in vitro assay. Originally, the in vivo data was used solely to complement the classic in vitro results (de Luise et al., '70).

The immediate antecedent of the in vivo competitive binding approach resides in the in vitro approach (Bergeron et al., '77). Some of the basic criteria for hormone receptor-interaction determined by the in vitro approach, that is dose-response, dose-displacement, saturability, affinity, and specificity, were verified with the in vivo approach (Bergeron and Posner, '79).

Similar to the in vitro assay, the in vivo method utilizes the interaction of receptors, radiolabeled hormone, and unlabeled hormone. As in the in vitro approach, the main principle is that of competition between labeled and unlabeled hormone for receptor occupancy, this time injected simultaneously, in animals refered to as **Controls.**

In this approach, the labeled hormone is allowed to circulate for a short period of time in order to investigate the early hormone-

receptor interaction. Bound hormone is separated from unbound by intracardiac perfusion initiated by washing with lactated Ringer's solution. The radioactivity retained in the tissues is determined as disintegrations per unit time per unit weight of tissue with a gamma counter and estimated in radioautographs as silver grains per area, per given structure, or per individual cell, in which case resolution is maximal.

The binding may be estimated exclusively in light and electron microscope radioautographs. Data obtained from animals injected with radiolabeled hormone is compared to the data obtained in animals co-injected with an excess of related or unrelated unlabeled hormone. Total binding is the radioactivity counted in radioautographs obtained from animals injected with radioactive hormone alone. Non-competitive binding is the radioactivity remaining in tissues of animals co-injected with excess unlabeled related hormone, and by analogy to the in vitro system, is referred to as non-specific binding. Competitive binding is that binding which is blocked by the co-injection of excess unlabeled hormone, and by analogy to the in vitro system, is called specific binding. Specificity is established using Control animals co-injected with excess unrelated unlabeled hormone.

Osteopetrosis

Nomenclature and classification

The disorder was originally named after Albers-Schonberg, a german radiologist who, in 1904, gave the first clear description of the condition after X-rays examination of the sclerotic bone obtained from a 26 years old man with generalized sclerosis of the skeleton and

multiple fractures. In 1921, it was refered to as **marble bone** by Schulze and Karshner and in 1922, in a radiological review, they introduced the general designation **osteopetrosis** (see review in Johnston et al., '68).

In 1936, based on clinical observations which suggested variability among human cases, McPeak proposed to subdivide osteopetrosis into malignant and benign types. In 1964, Rubin emphasized the early development of complications, by using the terms congenital and tarda, referred to in the Paris nomenclature as **precocious** and **delayed.** Today, in humans, osteopetrosis is diagnosed as osteopetrosis with precocious manifestations (congenital, malignant, autosomal recessive form), or osteopetrosis with delayed manifestations (tarda, benign autosomal dominant form). Although most cases are identified as one of the above, the numerous atypical cases suggest heterogeneity within each of these categories.

Transmission of the condition

Osteopetrosis may be induced in birds by infection of the osteoblasts with retrovirus MAV. 20, resulting in a selective acceleration in bone formation (Boyde et al., '78), or it may be genetically transfered in an autosomal recessive fashion to the offspring of many species of mammals including rodents, rabbits, dogs and cows (see review in Marks and Walker, '76).

Description

The condition is recognized at first by retarded skeletal growth. The skeleton is bulky, the long bones are shorter, clumsier in outline, with clubbing at the growing ends, and pathological fractures are common.

Generalized symmetrical sclerosis, revealed by opaque dense areas of bone in X-ray examination, may be found throughout the skeletal system, but are most prominent in the metaphyseal ends of long bones.

In the young, the original dense primary spongiosa remain long after birth. It is visible within the vertebral bodies and often results in abnormally small marrow spaces. In an effort to compensate for the obliteration, the condition is accompanied by a various degree of ectopic bone marrow formation referred to as dysmopoesis and manifested by hepatosplenomegaly (see review in Marks, '84).

The absence of teeth in these mutants is believed to result from lack of bone resorption in the dental crypt. The persistence of hanging ledges to the crypt hypothetically interferes with the normal eruption process. Dentinogenesis appears abnormal (Doykos, '67).

Defective bone resorption and remodeling

Possible primary endocrine disorder

Parathyroid hormone and calcitonin, two hormones known for their regulatory role in bone metabolism and remodeling (see reviews in Gothlin and Ericsson, '76 ; Holtrop and King, '77), have been considered for their possible involvment in the generation of this condition (see review in Marks and Walker, '76). The possibility of an endocrine related disorder was suggested by the observation that the grey lethal gl/gl osteopetrotic mouse tolerated doses of parathyroid extract (PTE) that would have killed normal littermates (Barnicot, '45). Attempts to cure or produce the disease on this endocrinological basis have, with an exception in a human case (Glorieux et al., '81), failed, sugesting that osteopetrosis does not result from primary endocrine inbalances.

Target cell disorder

Osteoclasts are the active cells in bone resorption and remodeling. Significant alterations in cell number, size, ultrastructure, as well as in enzymatic activity, have been documented (Marks and Walker, '76). Moreover, the disease is cured in many cases by operations whereby a source of competent osteoclast precusors are provided either by temporary parabiosis (Walker, '72) or by bone marrow and spleen cellular transplants (Walker, '75b; Marks and Walker, '76). It is thus reasonable to consider the possibility of a disorder at the cellular level.

Others

The inborn error of metabolism does not reside in the calcified tissue itself. This has been shown in transplantation experiments, where osteopetrotic grey lethal (Barnicot, '41), and microphthalmic (Ossipov and Konyukhov, '67) bone transplants are resorbed when transplanted into normal littermates.

When present, the osteoclasts appear inactive with poorly or abnormally developed ruffled borders (Marks, '73; Holtrop et al., '81). A reduction (Barnicot, '47; Marks, '82), as well as an increase in osteoclast number (Marks, '73), has been reported. Lysosomal production is decreased or when normal, the lytic enzymes are not secreted at the bone-cell interface (Marks and Walker, '76). Besides the obvious and well demonstrated involvement of inadequate bone resorption in the pathogenesis of osteopetrosis, abnormalities of a non-skeletal nature including alteration in host defense, hemopoiesis, and macrophage biology, have been documented (see review in Marks, '84). The condition has also been associated with systemic and renal tubular acidosis (Vainsel et al., '72; Whyte et al., '80).

Osteopetrotic models

Osteopetrotic op/op mice

The osteopetrotic op/op mutant mouse was discovered in 1970, at the Jackson Laboratory, in Maine. It was described in the scientific literature in 1976 as a recessive skeletal mutation on chromosome 12 (Marks and Lane, '76).

Generally speaking, the mutant mice are smaller than their phenotypically normal littermates as their growth is significantly retarded. Among the frequent skeletal deformities is the development of s-type curves in the tail. The head is domed and the limbs are shorter than normal. The absence of incisors is the most reliable external criteria for identification.

The parafollicular cells in the thyroid gland are significantly increased, the serum level of calcitonin is normal with a slight increase in PTH serum level (see review in Marks, '84). The model is normocalcemic and hypophosphatemic.

Bone formation, as measured by ^{3}H-proline incorporation is at first increased, then decreased at 6 weeks of age. A reduction in PTE-induced hypercalcemia and bone resorption has been reported (Marks, 82). The interior of the long bone is filled with primary spongiosa, and a definite marrow cavity appears only late in development.

The osteoclasts are generally smaller and reduced in number (Marks and Walker, '76). The acid phosphatase content is decreased and abnormally distributed (Marks and Lane, '76). Hypertrophy of the clear zone and ruffled border has been observed in the osteopetrotic osteoclast (Marks, '84).

Remission occurs at 90 days (Lane, '75) and the condition cannot be cured by bone marrow or spleen transplants (Marks et al., '84).

Among the abnormalities of a non-skeletal nature, inadequate host-defense response is suggested by a reduction in response to T and B cell mitogens. Cell suspensions of the spleen show an elevated baseline proliferative response (Olsen et al., '78; Milhaud and Labat, '78; Minkin et al., '82). There is a reduction in resident blood monocytes and peritoneal macrophages, and an increase in the ability of extramedullary hemopoietic tissue to form in vitro monocyte-macrophage colonies (Wiktor-Jedrzejczak et al., '81).

Large lipoid masses, in vascular and extravascular locations, have been observed (Marks and Lane, '76). Moreover, the osteoclasts contain unusual toluidine blue positive and electron dense cytoplasmic inclusions. Electron microscope observations suggest that they are lipoprotein in nature (Marks, '82).

Microphthalmic mi/mi mice

Hertwig, in 1942, first described the microphthalmic mi/mi mouse. The model is characterized by a complete absence of pigment, a distorted skeleton, and a reduction in size as a consequence of general retardation in development. Marrow cavities fail to develop and the incisor teeth do not erupt (Hertwig, '42). Poor tooth development was attributed to abnormal growth, whereas the non-eruption of the molars and incisors was attributed to a disturbance in the growth of the actual tooth rudiments, as well as absence of bone resorption (Konyukhov and Ossipov, '66). However, absence of bone over the first molar is not accompanied by eruption, and when the teeth erupt, they are always smaller than normal,

and the volume of pulp in the microphthalmic teeth is considerably less than in normal littermates.

A 6 fold increase in C cells of the thyroid has been reported (Marks and Walker, '69). The basal level of calcitonin is normal (Kent and Cohn, '81), but calcitonin-induced cAMP production is decreased by a three fold factor in the microphthalmic model. A small increment, in the circulating PTH (see review in Marks, '84) has been reported, and introduction of PTE in the system does not result in hypercalcemia (Marks, '77). The ability of CT to inhibit PTH-stimulated hyaluronate synthesis was impaired (Kent et al., '79). The model is hypocalcemic (Walker, '66; Marks and Walker, '69) and hypophosphatemic (Murphy, '73).

Bone matrix formation, measured in vivo as the rate of ^{3}H-proline incorporation, is elevated in the mi/mi mutants together with citrate and alkaline phosphatase (Marks, '77). Histologically, this is supported by the presence of numerous osteoblasts and increased thickness of osteoid tissue (Marks and Walker, '69). There is a 50-95% reduction in PTH-stimulated bone resorption , as measured by the rate of ^{3}H-hydroxyproline release (Wong et al., '83; see review in Marks, '84). However, in in vitro culture studies, the microphthalmic osteolytic ability appears increased (Marks, '74). In the mi/mi mouse, there is bone matrix resorption, but no mineral resorption (Jilka and Cohn, 83).

While not deficient in number, the size of the osteoclasts, as measured by electron microscopic morphometry, is greatly reduced with fewer nuclei and less cytoplasmic volume. The cytoplasm is not fully differentiated, the clear zone smaller, and a normal ruffled border is absent or insignificant (Holtrop et al., '81; Marks and Walker, '81). The mutants have an elevated blood level of alkaline phosphatase

(Murphy, '73) and a decreased osteoclast acid phosphatase production (Marks and Walker, '76). Although more recent reports support the view that the lysosomal enzymes and the collagenase content are not altered in these mutants (Holtrop et al., '81).

About 1 out of 4 microphthalmic mice live beyond the third month, and resorption is restored following establishment of cross circulation with a normal littermate, or bone marrow and spleen transplants (Walker, '72; Walker, '75b). High levels of exogenous PTE do not cure the disease.

As in the op/op mice, abnormal host defense response is suggested by a reduced response to B and T cell mitogens (Minkin et al, '82). The peritoneal macrophages elicited from mi/mi mice are not as chemotactically responsive to EAMS (Endotoxin Activated Serum) as cells from their phenotypically normal littermates (Minkin, '81). A reduction in phagocytosis has been observed as well (Chamber and Loutit, '79). There is a deficiency in the natural killer cells and a reduction in mast cells (Seaman et al., '79)

Osteosclerotic oc/oc mice

The osteosclerotic oc/oc mouse was reported in 1965 by workers at the Jackson Laboratory (Dickie, '66), and represents a recessive skeletal mutation on chromosome 19 in the mouse (Marks et al., '85). Affected animals are detectable at 10-12 days by their abnormal cycling behavior as they have a tendency to rotate on a particular side, abnormal shape of their feet, and the absence of teeth.

There is a 6 fold increase in the C cells of the thyroid gland (Marks and Walker, '69). An increase in circulating PTH has been reported and the model is hypocalcemic (see review in Marks, '84).

Skeletal preparations reveal extra deposits of bone at the chondrocostal junction, and in the hind limbs and ^{3}H-proline incorporation in bone is slightly elevated (Marks and Walker, '69), The increase in bone mass is believed to be the result of inadequate secondary bone resorption.

In this model, the size and number of osteoclasts are increased (Marks, C.R., cited in Marks, '84) but the ruffled border, hallmark of active resorption, is absent and acid phosphatase activity is greatly decreased in most osteoclasts (see review in Marks, '84). In an in vitro system, the oc/oc osteoclasts show an increase in bone resorbing activity (Marks, '74).

Most animals die by 30-40 days of age, and the disease cannot be cured by bone marrow or spleen cell transplants (see review in Marks, '84).

Incisor absent ia/ia rats

Osteopetrosis has been described in the incisor absent rat with marked effects on the teeth and on the skeleton (Greep, '41;

Schour et al., '49; Bhaskar et al., '54). In the long bones, spongiosa occupies most of the marrow cavities.

The model is normocalcemic, and there is a normal amount of parafollicular cells (Marks, '73). Hypophosphatemia persists long after birth (Kenny at al., '58).

Bone matrix formation is decreased slightly and neither the osteoblasts, nor osteoid tissue, are in excess. Bone resorption is decreased by a 35% factor, then, upon spontaneous remission of the condition, increased by a 20% factor (Marks, '73). In vitro bone resorption is 30% greater than that in normal littermates together with an excessive release of acid phosphatase (Marks, '74). PTH affects adenyl cyclase activity and lactic acid production to the same extent as in the bone of normal littermates (Rodan et al., '78). Serum calcium levels in young ia/ia rats is much less responsive to exogenous PTE than those of normal littermates (Marks, '73).

Osteoclasts although normal in size, distribution, and structure, are greater in number with absent or poorly developed ruffled borders (Marks, '73). Proliferation of the osteoclast precursors, and osteoclast formation proceed at approximatly twice the rate of that in normal littermates (Miller and Marks, '82). Excessive aryl sulfatase, and trimetaphosphatase (Schofield et al., '74) as well as excess acid phosphatase (Handelman et al., '64) have been reported. Although there is an increase in the acid phosphatase content, the enzyme is not liberated at the cell-bone interface, at least early in the mutant's life (Marks, '73). The latter suggests that they are capable of phosphatase synthesis, but unable to release it at the bone-cell interface.

Recovery from its resorptive failure occurs spontaneously though very slowly, with reestablishment of an extracellular secretion of lytic enzymes. However, it was claimed that normal ruffled borders are never observed and the sclerotic methaphysis remains throughout life (Marks, '73). Bone resorption can be restored following parabiotic union with normal littermates (Walker, '72) and by bone marrow and spleen transfers (Marks, '76; Marks and Schneider, '82).

There is no evidence of immunological incompetence. Although phagocytosis by ia/ia macrophages is unimpaired, and illicited macrophages are greatly reduced (Schneider et al., '81; Seifert, '84). The serum obtained from these mutants does not inhibit resorption, suggesting that the causative factor in this condition is not humoral (Raisz et al., '81).

PART II

MATERIALS and METHODS

The In Vivo Competitive Specific Binding Method

The in vivo specific binding approach, coupled with light and electron microscope radioautography, has been used to study the binding of ^{125}I-bPTH (Table 1), and ^{125}I-sCT in normal rats (Table 2) and in normal mice (Table 3). Calcitonin binding has been characterized in the sclerotic osseous tissue in four different osteopetrotic mutant rodents (Table 4).

The in vivo methods used for the work presented in this thesis involved the following technical procedures. Freshly, iodinated hormone was separated into three equal portions, and prepared according to the following protocol for intravenous injection. One of the portions consisting of radiolabeled hormone only, was injected into an animal refered to as the **Experimental** animal. The two other portions were co-injected with a 200-500 fold excess of related, or unrelated unlabeled hormone, into animals referred to as **Control.**

The successful use of the in vivo competitive specific binding approach required labeling of an appropriate quantity of high specific activity, biologically active hormone, and its adequate introduction into the system to be studied. Animals were sacrificed by whole-body perfusion-fixation, initiated with a lactated Ringer's solution to remove unbound hormone from the system. This permitted adequate preservation for accurate identification of the structures binding the labelled hormone in various tissues.

TABLE 1

Summary of Protocols
for Experiments with Bovine Parathyroid Hormone* in Normal Rats***

Experimental Designation	Sex	Weight g	**	Material injected	SA μCi/μg	Labeled Hormone μg	Excess Unlabeled Hormone μg	dpm X10^6 /0.1cc
BPTH(1-34)	M	100	E	^{125}I-bPTH(1-34)	455	.200	-	200
EXP 1	M	97	C	.....+ bPTH(1-34)		.200	100	200
BPTH(1-34)	M	58	E	^{125}I-bPTH(1-34)	493	.220	-	240
EXP 2	M	58	C	.....+ bPTH(1-34)		.220	50	240
BPTH(1-34)	M	50	E	^{125}I-bPTH(1-34)	410	.204	-	184
EXP 3	M	50	C	.....+ bPTH(1-34)		.204	50	184
	M	50	C	.....+ insulin		.204	50	184
BPTH(1-34)	M	85	E	^{125}I-bPTH(1-34)	475	.190	-	200
EXP 4	M	85	C	.....+ bPTH(1-34)		.190	100	200
BPTH(1-34)	M	90	E	^{125}I-bPTH(1-34)	735?	.124	-	200
EXP 5	M	88	C	.....+ bPTH(1-34)		.124	50	240
BPTH (1-84)	M	38	E	^{125}I-bPTH(1-84)	200	.499	-	240
EXP 6	M	40	C	.....+ bPTH(1-84)		.499	100	240

* The bovine parathyroid analogue [Nle-8,18, tyr-34]bPTH-(1-34)NH_2.
** E, Experimental animal injected with radiolabeled hormone **only**.
C, Control animal **co-injected** with an excess of unlabeled related hormone.
*** Sherman white rats obtained from the McGill University colony with the exception of EXPs 4,5. In these cases, Sprague-Dawley rats were obtained from Canadian Breeding Farm.

TABLE 2

Summary of Protocols
for Experiments with Salmon Calcitonin in Normal Rats.***

Experimental Designation	Sex	Weight g	**	Material injected	SA μCi/μg	Labeled Hormone μg	Excess Unlabeled Hormone μg	dpm X10^6 /0.1cc
SCT	M	109	E	^{125}I-sCT	562	.114	-	180
EXP 1	M	108	C	.....+ sCT		.112	50	140
SCT	M	100	E	^{125}I-sCT	645	.119	-	164
EXP 2	M	110	C	.....+ sCT		.118	50	162
SCT	M	96	E	^{125}I-sCT	712	.326	-	510
EXP 3	M	94	C	.....+ sCT		.326	125	510
SCT	M	92	E	^{125}I-sCT	735	.246	-	400
EXP 4	M	84	C	.....+ sCT		.246	125	400
	M	88	C	.....+bPTH(1-34)		.246	125	400
SCT	M	61	E	^{125}I-sCT	737	.186	-	300
EXP 6	M	65	C	.....+ sCT		.186	110	300
SCT	M	80	E	^{125}I-sCT	710	.082	-	128
EXP 7	F	65	E	^{125}I-sCT		.082	-	128
	F	80	C	.....+ sCT		.082	50	128
	M	65	C	.....+ insulin		.082	50	128

** E, Experimental animal injected with radiolabeled hormone **only**.
C, Control animal **co-injected** with excess unlabeled hormone.

*** Sherman white rats, obtained from the McGill University colony, were used in all cases.

TABLE 3

Summary of Protocols

for Experiments with Salmon calcitonin in Normal Mice ***

Experimental Designation	Sex	Weight g	**	Material injected	SA μCi/μg	Labeled Hormone μg	Excess Unlabeled Hormone μg	dpm X10^6 /0.1cc
SCT	F	22	E	^{125}I-sCT	500	.152	-	168
EXP 1	F	24	C	.....+ sCT		.152	150	168
SCT	F	25	E	^{125}I-sCT	710	.082	-	128
EXP 2	F	25	C	.....+ sCT		.082	50	128
SCT	F	25	E	^{125}I-sCT	710	.082	-	128
EXP 3	F	25	C	.....+ sCT		.082	50	128

** E, Experimental animal injected with radiolabeled hormone **only**.
C, Control animal, **co-injected** with an excess of unlabeled related hormone.

*** EXPs 1 and 2 (C57Bl/6), EXP 3 (CBA)

TABLE 4

Summary of Protocols ***
for Experiments with Salmon Calcitonin in Osteopetrotic Mutant Mice.

Experimental Designation	Sex	Weight g	Genetic background & nomenclature of carriers	**	Material injected	SA μCi/μg	Labeled Hormone μg	Excess Unlabeled Hormone μg	dpm X10^6 /0.1cc
SCT	F	14	B6C3, op/op	E	^{125}I-sCT	712	.028	-	170
EXP 1	F	22	B6C3, +/?	C	^{125}I-sCT		.028	-	170
OP Mice	F	24	B6C3, +/?	C	.....+ sCT		.028	27	170
SCT	M	24	B6C3, op/op	E	^{125}I-sCT	500	.152	-	168
EXP 2	F	24	B6C3, op/op	C	.....+ sCT		.152	150	168
OP Mice	M	26	B6C3, +/?	E	^{125}I-sCT		.152	-	168
	F	26	B6C3, +/?	C	.....+ sCT		.152	150	168
	F	26	B6C3, +/?	C	.....+ bPTH(1-34)		.152	165	168
SCT	F	6	C57Bl/6, mi/mi	E	^{125}I-sCT	710	.038	-	60
EXP 3	F	7	C57Bl/6, mi/mi	C	.....+ sCT		.038	50	60
MI Mice	M	12	C57Bl/6, +/?	E	^{125}I-sCT		.038	-	60
SCT	F	5	B6C3, oc/oc	E	^{125}I-sCT	710	.038	-	60
EXP 4	F	7	B6C3, oc/oc	C	.....+ sCT		.038	50	60
OC Mice	F	5	B6C3, +/?	E	^{125}I-sCT		.038	-	60
SCT	F	28	Long-Evans, ia/ia	E	^{125}I-sCT	710	.038	-	60
EXP 5	F	30	Long-Evans, ia/ia	C	.....+ sCT		.038	50	60
IA Rats	F	30	Long-Evans, ia/+	E	^{125}I-sCT		.038	-	60

** E, Experimental animal injected with radiolabeled **alone.**
C, Control animal, **co-injected** with an excess of unlabeled related hormone.

*** Mutant genetic background and nomenclature of carriers of rodents are indicated for each group.

Histological preparations, in particular the sectioning and radioautographic processing of the samples, were rigorously standardized. Quantitation procedures were rigidly applied, as the main aim of this method was to compare the radioautographic reactions obtained in **Experimental** and in **Control** animals.

Iodination of hormones

The procedures involved in iodination of protein were, labeling, purification of the labeled protein, and removal of the liquid phase prior to reconstitution for introduction to the selected system.

The choice of a method was dictated by the amino acid sequence of the molecule to be iodinated, and the requirement for biological activity and specific activity. During the labeling step, to introduce the radioactive iodide into the protein, the isotope was converted from iodide (I^-) to iodine (I_2) (Teitelbaum, '83). The active (I_2) was prepared just before the iodination by oxidation of the (I^-) with various oxidizing agents. During this process, the oxidizing agent not only oxidized the iodide but also some of the amino acid residues among which was methionine in the protein to be iodinated. Human calcitonin has one such residue in position 8 and bovine parathyroid hormone has two in positions 8 and 18. Oxidation of these would result in a loss of biological activity (Rasmussen and Craig, '62; Tashjian et al., '64).

The use of molecules, which contained no methionine residues, such as salmon calcitonin, or oxidation resistant analogues, among which was the synthetic sulfur-free analogue of the biologically active amino-terminus of bovine parathyroid hormone, were solutions to the problem of undesirable oxidation (Rosenblatt et al., '77). In the bPTH analogue,

isosteric norleucine residues, which could not be oxidized, were substituted for methionines in position 8, and 18 of the amino-terminal 1-34 fragment of the molecule. Also, a tyrosine amide residue was included in position 34, instead of the naturally occuring phenylalanine to facilitate iodination. Hence, highly oxidizing conditions, as in Chloramine T method, have been used to iodinate the resulting compound [Nle8,18, Tyr-34]bPTH-(1-34)NH_2 with no loss of biological activity.

The iodination of intact bovine parathyroid hormone (bPTH(1-84)) was accomplished with hydrogen peroxide, a mild oxidizing agent, in the presence of lactoperoxidase. The mild oxidation precluded the undesirable oxidation of the methionines in positions 8 and 18 of this molecule. The problem of employing milder oxidation conditions was that relatively few molecules of proteins were labeled, resulting in a preparation of low specific activity (S.A.). The S.A. could be increased by removing the unlabeled molecules using reversed-phase liquid chromatography, a process which separated the molecules on the basis of their hydrophobicity. ^{125}I-bPTH(1-84), being more hydrophobic, was retained for a longer time on the column than were the noniodinated molecules.

Iodination could also cause physico-chemical changes in the iodinated molecule (Hughes, '57). These changes varied with the protein iodinated and with the degree of iodination. Since in tracer studies, it was assumed that labeled molecules behave as unlabeled molecules, it was crucial to verify that no difference existed between the labeled and unlabeled species. Using bioassay techniques, it was determined if a radiolabeled hormone had retained biological activity.

The most convenient bioassay for PTH was the activation of the renal adenylate cyclase system *in vitro*. Biological activity of the ^{125}I-bPTH-(1-34) (Bergeron et al., '81) and that of bPTH-(1-84) (Kremer et al., '82) have been tested in this fashion. An alternative to verify the biological activity of PTH was the *in vivo* Munson bioassay in rats. It measured the ability of PTH to prevent the fall in serum calcium following thyroparathyroidectomy. This assay has also been used to test the biological activity of ^{125}I-bPTH-(1-34) (Unpublished data). The ultimate test for retention of biological activity of labeled hormone involved the concurrent assay of both labeled and unlabeled hormone in the system chosen, and the verification that both molecules induced the same response. Generally, *in vivo* bioassays require large amounts of hormone. Nevertheless, since the response to calcitonin in *in vitro* systems was too insensitive for use as a bioassay, the biological activity of labeled sCT was tested against that of unlabeled sCT by a method which tested the hypocalcemic action of both molecules *in vivo* (Warshawsky et al., '80).

Chloramine T iodination of sCT and [Nle-8,18, Tyr 34,]bPTH(1-34)NH_2

Salmon calcitonin (sCT[1]), and the analogue of the NH_2-terminal fragment of bovine parathyroid hormone ([Nle-8,18, Tyr-34]bPTH-(1-34)NH_2[2]), were iodinated by the Chloramine-T method (Greenwood et al., 63). The procedure involved the reaction of the carrier free iodide 125 (^{125}I-Na[3]) with the protein in a 1:1 molar ratio, in the presence of Chloramine T, a strong oxidizing agent.

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1. Synthetic salmon calcitonin (sCT), MW 3432, 2 500 mU/µg, obtained from Armour Sandoz Ltd., Basel, Switzerland, was available in powder form and was reconstituted to a final concentration of 100 µg of sCT in 100 µl of 10.0mM acetic acid.

2. Synthetic bovine parathyroid hormone the analogue of the biologically active NH_2-terminus of the bovine hormone ([Nle-8,18, Tyr-34]bPTH-(1-34)NH_2 MW 4073, was obtained from Bachem Corp. Torrance, CA. BPTH-(1-34), MW 4109, was obtained from Beckman Instruments, Spinco division, Palo Alto, CA.It was reconstituted to a final concentration of 100 µg in 100 µl of 0.2M phosphate buffer.

3. Sodium iodide 125 ($Na^{125}I$), S.A. 17 µCi/mg, obtained from New England Nuclear, was diluted in 0.5M phosphate buffer to a final concentration of 1 mCi per 10 µl of 0.2M phosphate buffer pH 7.5.

Presumably upon addition of the Chloramine T[1], a complex of iodine was formed with the sulfonamide in which the iodine carries a positive charge (Hunter,'66). The positively charged iodide molecule was an electrophile which preferentially reacts with the tyrosine in position 22 of the sCT and in position 34 in the [Nle-8,18, Tyr-34]bPTH-(1-34)NH_2 analogue on the carbon ortho to the negatively charged oxygen. The iodination was terminated by addition of sodium metabisulfite[2], a reducing agent, which converted any active iodine into iodide.

The pH of the solutions were slightly alkaline to partially ionize tyrosine residues. It was also desirable to use a small reaction volume to maximize the concentration of the reactants.

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1. Chloramine T The sodium salt of N-monochloro-p-toluenesulfonamide (ChT), $C_7H_7ClNO_2SNa.3H_2O$, MW 281.68, was obtained from Eastman Kodak Co. 5 mg of ChT was dissolved in 5 ml of phosphate buffer 0.2M pH 7.4 to give a final concentration of 1 mg of ChT per 100 µl of phosphate buffer.

2. Sodium metabisulfite ($Na_2S_2O_5$), MW 190.10 was obtained from Fisher Scientific Co.. To get a final concentration of 1.52 mg/ml, 7.60 mg of sodium metabisulfite was dissolved in 5 ml of phosphate buffer 0.2M, pH 7.4.

Technique to prepare ^{125}I-sCT, and ^{125}I-bPTH(1-34)

In a 12 X 75 mm glass culture tube, 25 µl of 10mM acetic acid containing 25 µg of sCT, or 25 µl of phosphate buffer (0.2M, pH 7.4) containing 25 µg of the [Nle-8,18, Tyr-34]bPTH-(1-34)NH_2, 800 µl of phosphate buffer (0.2M, pH 7.4) were added and mixed gently by hand. Subsequently, 200 µl of phosphate buffer (0.5M, pH 7.4) containing 20 mCi of Na^{125}I were addded and mixed gently by hand, together with 200 µl of oxidizing solution containing 2 mg of ChT. Always shaking, iodination was allowed to proceed 30 seconds, after which 200 µl of the reducing solution containing 3.04 mg of sodium metabisulfite was added to terminate the reaction.

Before subsequent manipulation, the amount of isotope incorporated in the protein was determined by the trichloroacetic acid precipitation test. Upon addition of a sample of the iodination preparation, proteins, which showed only a slight solubility in 40 % trichloroacetic acid, were precipitated, while free iodide remained in the supernatant after centrifugation.

The fraction of the isotope incorporated into the protein was the ratio of the radioactive counts (assessed in a gamma scintillation counter) in the solid phase, which contained the labeled proteins, over the counts in the solid phase plus the counts in the liquid phase. Knowing the initial amount of iodide used, the total amount of radioactivity incorporated was thus determined.

The acid precipitation test was done again after the purification procedures and prior to the calculation of the specific activity of the material to be injected.

The specific activity (SA) was defined as the amount of radioactivity incorporated per unit weight of protein. It was usually expressed in μCi/μg. It was calculated according to the following equation:

$$(SA) = x\ mCi\ ^{125}I \times \frac{\text{cpm in solid phase}}{\text{cpm in solid + liquid phases}} \Big/ x\ \mu g\ \text{of protein}$$

If the incorporation was judged satisfactory, as soon as possible after the iodination the labeled hormone was isolated from the other reactants by an appropriate purification procedure. ^{125}I-sCT was purified by the QUSO technique, whereas ^{125}I-bPTH(1-34) analogue was purified by column chromatography.

Purification of ^{125}I-sCT

In the first step, SEMP buffer (0.02M Phosphate buffer, 0.12M Na Cl, 0.005M EDTA. 0.001% merthiolate, pH 7.0) and QUSO[1](microfine silica) were added to the mixture. QUSO adsorbed calcitonin, but not the free iodide, nor the damaged hormone fragments. In a second step, the free iodide was adsorbed to added anionic Resin[2]. The radioactive hormone was eluted from the QUSO by addition of an acetone/acetic acid[3] mixture. Centrifugation at 6,000 rmp of the mixture for 2 minutes, brought about three phases, the QUSO, the Resin and the free iodide, and the labeled protein in the acetone/acetic acid phase. The acetone acetic acid mixture containing the labeled protein was retained.

1.QUSO Microfine precipitated silica, QUSO G-32, MW 292.25, was obtained from Philadelphia Quartz, Co.. 20 mg of QUSO is mixed with 20 ml of SEMP buffer.

2.Resin Anionic Exchange Resin, analytical grade AG1-X10, 200-400 mesh, effective pore small size was obtained from Bio Rad Laboratory.

3.Acetone/acetic acid A mixture of 20%(v/v) acetone and 1%(v/v) acetic

acid was prepared.

Purification of [Nle-8,18, Tyr-34]bPTH-(1-34)NH_2

Purification was achieved by a technique involving a Biogel P10 column (Bio Rad Laboratory) which separated components on the basis of their size. Prior to the purification, urea[1] was added to the labeled solution to a final 4.0M concentration to dissociate any peptide aggregates. The P10 column was eluted with NH_4 acetate buffer[2] and collected in 4 drop portions. The material eluted in three peaks: a first peak contained the damaged peptides, a second peak contained labeled biologically active hormone, and a third peak was free iodide.

After purification, the liquid phase, consisting of the eluting buffer, was removed. If the liquid was aqueous, the sample was frozen in a tube in acetone and dry ice at -40°C, then lyophilized; if the liquid phase was acetone, it was evaporated with a stream of nitrogen.

For injection, the material was reconstituted in Tris/HCl buffer (50mM, pH 7.5) and 2% BSA, in the desired volume.

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1. Urea (NH_2CONH_2), MW 60.06 is prepared to a 8.0M concentration in distilled water and added in a 1:1 ratio to the iodination mixture for a final molarity of 4.0M.

2. Ammonium acetate buffer The elution buffer is an 0.1M ammonium acetate buffer pH 5.0 containing 2% PTH-free plasma. The pH is adjusted with acetic acid.

Lactoperoxidase iodination of bPTH(1-84)

Lactoperoxidase labeling was used to iodinate intact bovine parathyroid hormone (bPTH-(1-84)). The method involved the reaction of carrier-free iodide-125[1] and bPTH-(1-84)[2] in a 1:4 molar ratio in the presence of hydrogen peroxide[3] a mild oxidizing agent, and lactoperoxidase[4] which served as a catalyst. The enzyme lactoperoxidase converted peroxide into water and oxygen. The iodine was polarized by its association with the oxygen of the peroxide, and reacted with the tyrosine residue in position 43 of the bPTH-(1-84) molecules. The reaction was terminated by the addition of the reducing agent dithiothreitol[5].

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1. Sodium iodide 125 ($Na^{125}I$), S.A. 17 μCi/mg, was obtained from New England Nuclear and was diluted in 0.05M phosphate buffer to obtain a final concentration of 1 mCi/10 μl of phosphate buffer (0.02M, pH 7.5).
2. Native bovine parathyroid hormone (bPTH-(1-84)), MW 9562 was obtained from Bachem in a powder form. It was mixed to a final concentration of 200 μg per 100 μl of 0.05M phosphate buffer pH 7.5.
3. Hydrogen peroxide (H_2O_2), 30.4% was obtained from Fisher and prepared at 1:100 000 part.
4. Lactoperoxidase Grade B, 20 310 mU/μg, was obtained from Calbiochem. A stock solution of 200 μg per 100 μl of 0.05M phosphate buffer pH 7.0 was prepared.
5. Dithiothreitol DTT, MW 154.3, was obtained from Sigma, prepared to give a final concentration of 148 mg per 100 μl of 0.2M Tris buffer pH 8.5.

Technique to prepare ^{125}I-bPTH(1-84)

In a 12 X 75 mm glass tube 100 µl of phosphate buffer (0.05M, pH 7.0) containing 200 ug of bPTH-(1-84), 200 µl of phosphate buffer (0.05M, pH 7.0) containing 20 mCi of $Na^{125}I$, 400 µl of phosphate buffer (0.05M), pH 7.0) containing 800 µg of lactoperoxidase were added and mixed gently by hand. Subseqently, 200 µl of H_2O_2. were added and iodination was allowed to proceed for 30 seconds of gentle mixing at room temperature. Afterwhat, 380 µl of Tris buffer (0.2M, pH 8.5) containing DTT, the reducing agent was added to terminate the iodination followed by a 60 minutes incubation at room temperature.

Purification of ^{125}I-bPTH(1-84)

If the percent incorporation of the isotope into this peptide was judged satisfactory, as estimated by the trichloroacetic acid precipitation test, purification of the hormone was acheived by reverse-phase liquid chromatography (Kremer et al., '82).

In this procedure, the labeled hormone was initially extracted using a cartridge of octadecylsilylsilica (ODS-silica) first wetted with 5 ml of 80%(v/v) acetonitrile containing 0.1% trifluoroacetic acid (CF_3COOH; TFA) and rinsed with 5ml of 0.1% TFA. After loading the mixture, 20 ml of 0.1% TFA was passed through, to remove the reactants of the iodination procedure and unused $Na^{125}I$. Elution of the labeled hormone was acheived using 3 ml of 80% acetonitrile containing 0.1% TFA in H_2O.

Cartridge eluates were injected onto a C_{18} µ Bondapack column in a Waters gradient liquid chromatography system, employed for high

pressure liquid chromatography[1]. The column was eluted at a flow rate of 1.5 ml/min for 30 minutes, with 40% acetonitrile in 0.1% TFA, and for 60 minutes, with 40-55% acetonitrile in 0.1% TFA. Eluted 1 ml fractions were collected in borosilicate glass tubes (12 x 75 mm). These tubes were monitored for UV absorbance at 214 nm , employing an LDC fixed wavelength UV monitor III model 1203 detector, and for radioactivity in a Packard gamma counter 51% efficiency. Radioiodinated hormone was eluted in a second gradient run. The elution pattern, determined by counting 1 ml fractions, was reproducible and allowed for a routine collection of a fraction containing monoiodinated bPTH-(1-84), which was free of residual unlabeled PTH. The material collected in 1 ml fractions was counted and a graph of the radioactivity content was plotted.

The solvent was poured out of the tube containing the iodinated hormone. Since the labeled hormone adhered to the walls of the glass tube, the tube containing adherent ^{125}I-bPTH-(1-84) was stored at -20°C. To recover additional hormone, the liquid phase was removed by lyophilization.

The hormone was recovered for use as a radioligand by the addition of PTH-free plasma to the tube and by diluting to the required volume in Tris/HCl buffer (50mM, pH 7.5).

1.ODS-Silica Octadecylsilylsilica, Sep-Pak C_{18}, Waters Associates, Mississauga, Ontario.

Animal procedures

The animal procedures involved in the performance of the *in vivo* competitive binding approach to study the hormone-receptor interactions consisted of anaesthesia, and intravenous injection of the radioligand followed by intra-cardiac wholebody perfusion.

Animals were anaesthetized by intraperitoneal injection of Nembutal (pentobarbital sodium 60mg/ml (Nembutal); 0.1%(v/w) for rats; 0.02%(v/w) for mice).

Five minutes after sedation, the right external jugular vein was exposed. The appropriately prepared freshly iodinated hormone was injected with a Glaspak disposable syringe (0.5cc, 26g 3/8 inch) and allowed to circulate for 2 minutes.

At the end of this period, animals were sacrificed by gravity perfusion through the left ventricle. The process was begun by simultaneous puncture of the right atrium and a 25 ± 5 second perfusion with lactated Ringer's at a rate of 12 ± 2ml/10secs for rats, and 3 ± 1ml/10secs for mice, to wash out all unbound hormone from the circulation. This was followed immediately by a 10 to 30 minute perfusion with a fixative solution of 2.5%(w/v) glutaraldehyde, in 25%(v/v) of a 0.2M Sorensen's phosphate buffer and 0.1%(w/v) sucrose, pH 7.3. The tissues of interest were dissected, cut in slices of appropriate size for tissue processing, and left over night in the fixative solution at 4^{o}C.

Processing of tissue samples

Decalcification of the mineralized tissues

Dissected long bones including humerus and tibia were divided in two along the longitudinal axis, packaged in individual cotton bags with proper labeling, and immersed in a decalcifying solution consisting in 4.13%(w/v) ethylenediamine-tetraacetic acid (EDTA) and 11.2%(w/v) sodium hydroxide pellets (Warshawsky and Moore, '66). The tissues were decalcified at 4°C for a period of 20±5 days, a length of time proven adequate for sufficient demineralization. Afterwards the decalcified tissues were trimmed anew to demonstrate the entire proximal epiphyseal plate and surrounding tissues. Prior to postfixation, dehydration, and infiltration, the tissues were left over night in 75%(v/v) 0.2M phosphate buffer to remove all EDTA from the tissue. Subsequently, the mineralized tissues were processed identically to the soft tissues.

Processing of decalcified and soft tissues

Tissue samples obtained from liver and kidney were rinsed in 6 changes of washing buffer 75%(v/v) 0.2M phosphate buffer and separated into two groups. One group was processed for light microscope radioautography, the other for electron microscope radioautography.

For **light microscope radioautography**, the tissues were postfixed in 1% osmium tetroxide in veronal acetate buffer pH 7.4 for 2 hours at 4°C, rinsed with phosphate buffer, and stored for dehydration.

For **electron microscope radioautography**, the tissues were postfixed in an aqueous solution of 1.5%(w/v) potassium ferrocyanide ($K_4Fe(CN)_6$ and 1%(w/v) osmium tetroxide (OsO_4) for 1.5 hour (Karnowsky, '71). They were then stained **en block** with 2% uranyl acetate

in NaHMaleate-NaOH buffer pH 4.2 for 2 hours at 4°C (Lewis and Knight, '77). Rinsing of the tissues was done with the NaHMaleate-NaOH buffer, prior and after block staining.

Dehydration for light and electron microscope radioautography consisted of three 10 minute changes of ethanol ranging from 70%(v/v) up to 100%. Infiltration consisted of three consecutive periods of 12 hours, in a solution of increasing Epon 812 concentration, and was preceeded by three 10 minute changes in propylene oxide. After a final 2 hours in Epon, the tissues were encapsulated and polymerized for 48 hours, in an oven set at 60°C.

Radioautographic techniques

Once injected into the systemic circulation of an animal, isotopically labeled, biologically active hormone was bound to receptors to initiate a series of events, resulting in the proper biological response. Radioautography consists of an elaborate technique which located in a very accurate way, the position of the radioactive molecules in adequately prepared histological specimens. The technique has its origin in 1946 when Leblond and Belanger first painted the photographic emulsion over radioactive sections placed on glass slides. The coating technique, now used, involves dipping the slides containing the radiolabeled sections into fluid emulsion. this method has been described in detail for both light (Kopriwa and Leblond, '62), and electron microscope radioautography (Kopriwa, '73).

Light microscope radioautography

Sectioning. After 48 hours of polymerization, blocks prepared for light microscope radioautography were trimmed to a block face, and 0.5 µm thick sections were cut with a glass knife on a Huxley ultramicrotome. Sections were aligned carefully on glass slides, dried on a hot plate at 80°C, and prestained with iron hematoxylin.

staining procedures. The prestaining procedure involved three steps. First, the glass slides containing the sections were put onto a hot plate set at 80°C, flooded with a mordant solution of 5%(w/v) ammonium sulfate for an adequate amount of time, then rinsed in several changes of distilled water. In a second step, the slides were flooded with Regaud's hematoxylin ((1%(w/v) in 10%(v/v) ethyl alcoholand 10%(v/v) glycerine)) for an amount of time equal to that of the mordanting, then rinsed again with distilled water. In a final step, the slides containing the stained sections are flooded with tap water for a period of three minutes, and rinsed again in distilled water. After this staining procedure, the slides are dried for at least 15 minutes on the hot plate to prevent lifting of the sections during the radioautographic processing.

Coating with the radioautographic emulsion. The glass slides containing the prestained radioactive sections were covered with NTB2 (Eastman Kodak) bulk emulsion. This emulsion was used because of the regular size of its silver grains, low background fog, and high sensitivity. Prior to usage, the flask containing the refrigerated emulsion was placed in a water bath at 40°C. When melted, the emulsion was poured carefully into a cylindrical staining jar (Borrel tube).

In the coating technique, the slides containing the radioactive sections were held at the frosted end, and dipped for one second in the melted emulsion. After withdrawal, the back of the slides were wiped with soft paper, and allowed to dry in a vertical position. Satisfactory preparations were obtained by slow drying with their base on tissue paper to absorb the excess emulsion at 28°C with 80% relative humidity at best. Once dried, the coated slides were placed in plastic boxes. Small bags made from tissue paper containing 15-25 gm of indicating Drierite, a drying agent, were added to the boxes prior to tightly sealing them with black adhesive tape. After exposure for an adequate amount of time, they were processed.

The processing of the exposed radioautographs involved development for 6 minutes in Kodak D 170 developer, passage through a distilled water bath, and fixation for 3 minutes in Eastman Kodak acid fixer, containing a hardener which prevented swelling and displacement of the emulsion. Finally, processed radioautographs were washed for 15 minutes in running tap water.

During the processing, the darkroom temperature was maintained at 17-18°C. Mounting of the coverslips was achieved using a drop of the embedding agent. The slides were put in an oven at 60°C for a day to polymerize. Before quantitation of the silver grains and photography, the radioautographs were cleaned in a 1:1 solution of ethanol and 1N hydrochloric acid to remove the emulsion from the back of the radioautographs.

Electron microscope radioautography

Sectioning. After polymerization, the blocks prepared for electron microscope radioautography, were trimmed to the area of interest.

Sections of silver-gray interference color were cut on a Reichert Om U2 ultramicrotome with a diamond knife and deposited on celloidin coated glass slides (Salpeter and Bachmann, 64). To prepare the **celloidin coated slides,** precleaned slides were wiped with Kimwipes, type 900S tissue paper, then with lens paper. The entire slide, with the exclusion of the frosted end, was dipped in 1%(v/v) celloidin in isopentyl acetate, then placed vertically on a tissue paper. The slides were dried overnight in a dust free cabinet.

As the thin sections were cut, they were picked up with a loop and deposited on the coated slides, without touching the celloidin film. This was accomplished by placing a V shaped wedge of filter paper from above at the edge of the loop and soaking up the water at the same time that the section made contact with the celloidin film. The slides were adequately labeled for identification, and the position of the groups of sections were marked on the back of the slides with an opaque adhesive paper circle.

The slides containing the sections were further coated with a thin vacuum evaporated carbon layer 50 Å thick. The carbon layer provided a good substrate for the spreading and adhesion of the emulsion.

Coating with the radioautographic emulsion. A semiautomatic coating device was used to spread IlfordL4 Nuclear track emulsion, as a uniform monolayer of silver bromide crystal. These crystals had a small diameter and high sensitivity. For application of the IlfordL4 Nuclear track emulsion, the temperature of the darkroom was adjusted to 22°C and a relative humidity of approximatly 50%. Similar to the procedure for light microscope radioautography, the emulsion was melted at 40°C

in a water bath, and transfered to the 32°C water bath of the semiautomatic coating instrument. The slides were attached to the device and , one at the time, they were manually lowered into the emulsion, then mechanically withdrawn from the solution at a low constant, adjustable rate. Upon removal, the slides were dried in a vertical position for 15 minutes on plastic racks.

The slides were stored as for light microscope radioautography and exposed for an appropriate amount of time, then developed. The type of developer, the developing time and the temperature determined the number, the size, and the shape of the visible silver grains. Routine development of L4 emulsion consisted of one minute in the developer, diluted 1:10 with distilled water. After development the radioautographs were rinsed in a stop bath of distilled water for 30 seconds, fixed for 2 minutes in 24% sodium thiosulfate, and finally washed in 5 changes of distilled water for 1 minute each. All the solutions for darkroom procedures were at 20°C. Immediately after a crucial 3 minute drying period at room temperature the radioautographs were transferred from the supporting slides to the EM grids. This transfer was accomplished by cutting a strip of the celloidin-sections-emulsion complex and very slowly immersing the glass slide below the surface of the dish of distilled water thereby floating off the celloidin film on which the sections were held. While the film was floating, a clean, flat 200-300 mesh EM grid was placed matte side down, on each group of sections. Using two moistened filter papers placed on an aspirating device connected to a vacuum pump, the floating grids were sucked up onto the filter paper, removed from the aspirator and dried sections up.

The grids containing the radioautographs were cleaned and stained. For this study 2 grids were prepared from 3 different blocks for each animal injected. One, held at the tip of a fine forceps was plunged repeatedly for 1-2 minutes in glacial acetic acid, the other was plunged 3-5 minutes in isopentyl acetate. The treatment with the acetic acid dissolved some of the gelatin of the emulsion, the treatment with isopentyl acetate removed some of the celloidin. All grids were stained for 5 minutes with lead nitrate 2.66%(w/v); and sodium citrate (3.52%(w/v) and 16%(v/v) 1N sodium hydroxide; pH 12 (Reynolds, '63).

Quantitation of the radioautographic reaction

Immediately after intracardiac perfusion-fixation, the tissues of interest were removed. Several samples were taken from the liver, the bones, and the kidneys. These were prepared for histological examination. From the samples examined, 3 blocks revealing adequate prewashing and fixation, were further sectioned and processed for light microscope radioautography. The radioactivity content was estimated by counting silver grains in the emulsion at 1 000X magnification over 0.5 µm thick Epon light microscope radioautographs exposed for an adequate period of time to reveal a sufficient, but countable number of silver grains.

Hepatic tissue. In liver tissue, the silver grains were counted and recorded per frame overlying a 10,000 μm^2 area of liver parenchyma (Fig. 1). In each animal, counts were taken in 15 such areas.

Skeletal tissue. In bone tissue, silver grains were counted and recorded per row of osteoblasts underlying a grid length of 100 µm (Fig 2), and per osteoclast profile underlying a grid surface of 900 μm^2 (Fig 3). The counts were obtained from cellular elements localized in the humerus and tibial metaphyseal region containing the entire proximal epiphyseal plate and surrounding tissue. In each animal silver grains were counted in 50 samples of osteoblast rows and osteoclast profiles.

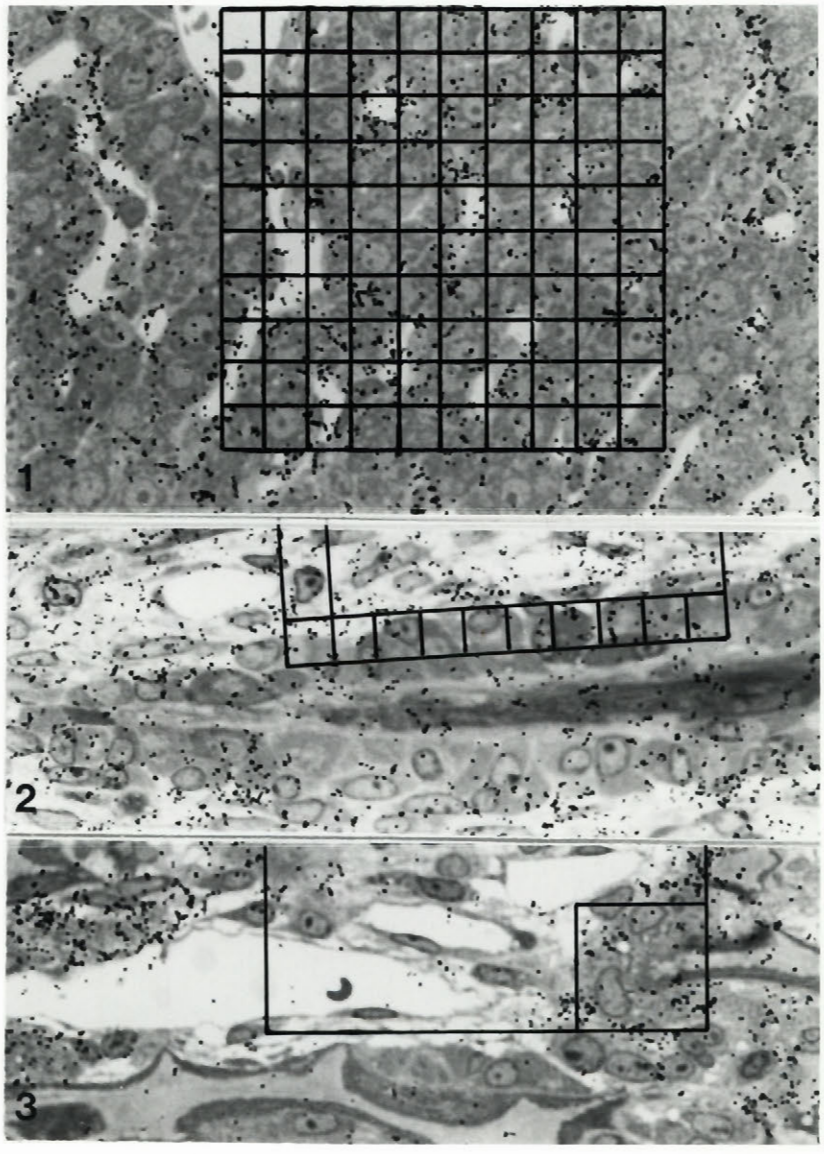
Renal tissue. Macroscopic examination of a bissected kidney revealed two main divisions: a broad outer zone of dark red substance, the cortex, and a paler inner zone, the medulla. The medulla could be subdivided further into a darker outer, and a lighter inner medulla.

PLATE 1
Illustration
of the method used to quantitate the radioautographic reaction in liver and osseous tissues

Grids used for quantitating the radioautographic reaction in liver tissue (Fig. 1) and in osseous tissue (Figs. 2,3) are shown. The radioautographic reaction was estimated by counting silver grains in 0.5 μm thick Epon radioautographs using an occular grid in a Carl Zeiss light microscope. Counts were recorded at X1,000 under oil immersion. X600.

Figure 1 — In liver tissue, silver grains were recorded per frame overlying a 10,000 μm^2 area of liver parenchyma devoid of obvious Kupffer's cell profiles.

Figures 2,3 — In osseous tissue, the silver grains were recorded by row of osteoblasts (Fig. 2) underlying a grid length of approximately 100 μm, and per osteoclast profile (Fig. 3) underlying a grid area of 900 μm^2.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The same divisions were recognized upon microscopic examination of histological sections with the additional resolution of two stripes in the outer medulla: an outer stripe, adjacent to the cortex, and an inner stripe, in contact with the inner medulla.

The kidney substance comprising approximately a million units of similar structures, develops from the junction of the metanephrogenic blastema with the uteric bud. The apparent zonation on a mid-cross sectional axis is mainly the result of similar units running in register all oriented in the same fashion.

In this study, hormone binding to the elements of metanephrogenic origin was examined. Radioautographic reactions associated with the renal corpuscule and with the tubular portions of the nephron have been estimated separately. The same divisions were observed in mice (Fig. 4) and in rats (Fig. 5).

The **renal corpuscle,** commonly referred to as the glomerulus, is composed of the glomerulus consisting of a cluster of capillaries together with the visceral and the parietal layers of Bowman's capsule.

Silver grains were recorded per 2 500 μm^2 areas of parenchyma composed of the endothelial cells of the glomerulus, the podocytes forming the visceral layer of Bowman's capsule, as well as mesangial cells. The standardized area was delineated using an occular grid, overlying any of the glomeruli found in the cortex (Figs. 4 and 5 A). For each animal silver grains were counted in 10 such areas.

Tubular morphology. The tubular portion of each nephron exhibited three continuous portions that revealed somewhat different structural features and arrangements. Each segment in the tubular structure was lined with a

Segmentation of the distal tubule. The earliest part of the distal tubule marked the boundary between the inner and the outer medulla. The latest part joined with cortical collecting duct elements. Similar to the proximal tubule portion of the nephron, it was customary to describe the distal tubule in terms of a straight ascending part or pars recta and a tortuous portion, the distal convoluted tubule. The distal convoluted tubule was considered to be that portion of the distal tubule beyond the macula densa. Structurally, it consisted of three descrete segments: the convoluted part of the distal tubule (DCT), the connecting tubule (CNT), and the cortical collecting duct (Kaissling, '80). As many as 5 structurally different cell types, with certain cell types occuring only in a given portion, have been described (Crayen and Thoenes, '78; Kaissling and Kriz, '79).

Type 1 cell, the **Mittelstuck** (Crayen and Thoenes, '78), or the distal convoluted tubule cell (DCT-cell) (Kaissling and Kritz, '79), displayed intense interdigitations and basal infoldings arranged perpendicular to the basal lamina. These ascended to approximatly three quarters of the cell height. Long mitochondria occupied these infoldings accounting for the basal striation seen in the light microscope. One or more nuclei lay in the apical portion of the cell, together with numerous small mitochondria. The apical cell membrane put out short microvilli, which were irregularly distributed. A small number of uncoated vesicles were found in the apical cytoplasm (Fig. 78).

The type 3, the Clear Cell (Crayen and Thoenes, '78) or the connecting cell (CNT-cell) (Kaissling and Kritz, '79), could be found adjacent to the DCT-cell or to the two cell types of the collecting duct. The CNT-

cell was rather electron-lucent. It was characterized by small shallow basal membrane infoldings and small roundish mitochondria scattered in a clear cytoplasm. Extensive membrane contact with mitchondrial membrane occurs only occasionally. Smooth endoplasmic reticulum was conspicuous, although polysomes were rarely found and a fair number of uncoated vesicles were observed in the apical cytoplasm. In rats, the clear cell processes interdigitated consistently and reached far beneath neighbouring cells, consequently, some clear profiles of that cell may be observed among darker cells. The apical surface had short microvilli (Fig. 73).

Type 4 or intercalated or dark cell of the collecting duct was first described by Novikoff in 1959. Many reports on their ultrastructure have appeared since then (Crayen and Thoenes, '78; Kaissling and Kirtz, '79; Nicholson and Kendall, '83). The cell was characterized by its electron dense cytoplasm and a very conspicuous accumulation of apical electron lucent membrane bound vesicles. The mitochondria were numerous and larger than in the clear cell. They were found throughout all of the cytoplasm, together with a large Golgi complex, and abundant smooth endoplasmic reticulum. The luminal surface had relatively long microvilli which were often grouped together in tuft-like formations which were responsible for the characteristic stripes when viewed with the scanning electron microscope (Dobyan and Bulger, '80; Fig. 79).

In the present investigation, the silver grains were recorded over the following segments in the distal tubular nephron.

Count were taken in the **ascending thick limb** (AL) found in the inner (MAL I_s; Fig. 4E), and outer (MAL O_s; Fig. 4F) stripes of the outer medulla, as well as in the cortex (CAL; Fig 4G). In the cortex, cross sections of the thick ascending limb were recognized because the entire thick ascending limb was made of DCT-cell exclusively and the tubule had a relatively smaller diameter.

Cross sections of the **distal convoluted tubule** (DCT) were found in proximity to a glomerulus in the cortex. They consisted of the earliest part of the distal convoluted tubule (DCT) which started abruptly shortly after the macula densa. A cross section of that segment was recognized by its large diameter and was homogeneously composed of the characteristic DCT-cells. In that segment the DCT-cells reached their maximal height and nuclei were found in the apical portions of the cells (Fig. 4H)

The distal convoluted tubule (DCT), of metanephrogenic blastemal origin, was joined to the collecting duct, of uteric bud origin, by an intermingling of elements arising from the two primordia. Consequently, **the connecting segment** (CNT) was characterized by cellular heterogeneity. The first appearance of a clear cell ,also called CNT-cell, which gradually replaced the DCT-cells, was regarded as the beginning of the connecting tubule. The connecting tubules were lined by DCT-cells, CNT-cells and intercalated cells (Fig. 4J).

The last part of the distal tubular portion of the nephron has been customary called arched collecting duct or pars arcuata to differentiate it from the straight collecting duct. The first appearance of the principal cell of the collecting duct system, and the

intercalated cells between CNT-cells defined the beginning of the cortical collecting tubule (ACT). Cross sections of this portion of the distal tubule were located in the cortex. They had a small diameter and their walls did not contain any DCT-cells.

PLATE 2

Illustration of the method used to quantitate the radioautographic reaction in kidney tissue

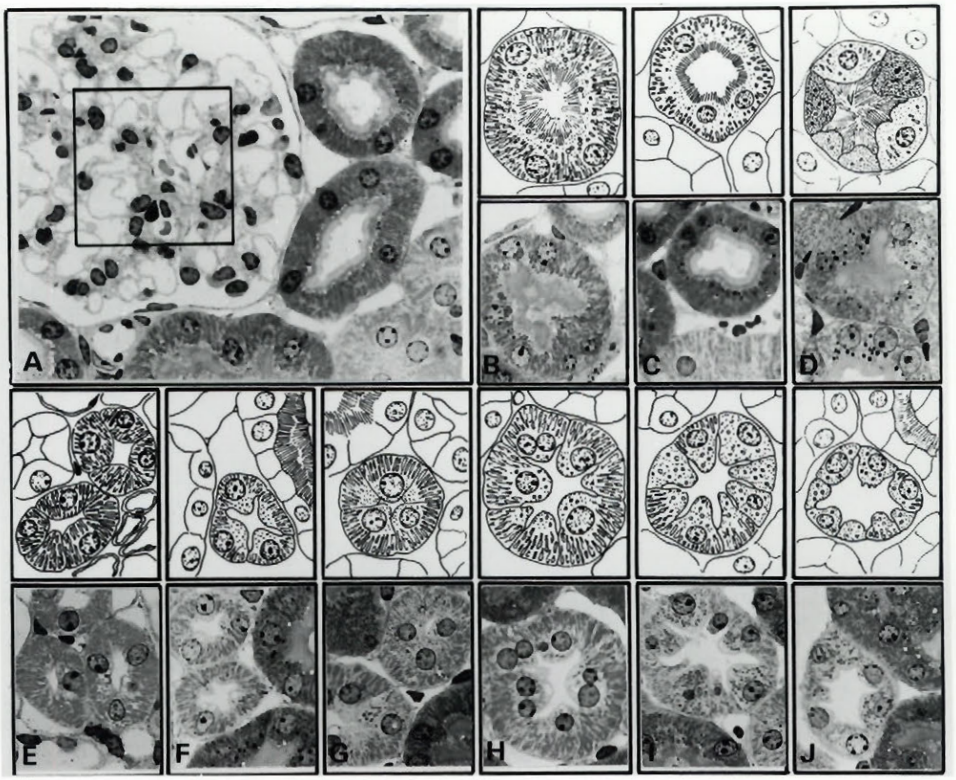
Segmentation used for quantitating the radioautographic reaction in the nephrons of the mouse (Fig. 4) and of the rat (Fig. 5).

In an attempt to determine exactly which part of the nephron is the site of hormone binding, renal corpuscle, proximal and distal tubular segment reactions were estimated. Counts were made at X1,000 oil immersion, in 0.5 μm thick Epon radioautographs. X450.

Figures 4,5 (A) Renal corpuscle
Silver grains were recorded over the renal corpuscles per 2,500 μm^2 area delineated with an occular grid (Fig. 4A)

Figures 4,5 (B-D) Proximal tubule
In the tubular portion silver grains were recorded per cross section in the different segments of the tubular portion of the nephron (Figs. 4, 5; (B-J)), identified according to their histology, and position. In Epon radioautographs the cross section were identified as a section in segment 1 (B), segment 2 (C), or segment 3 (D) of the proximal tubular portion of the nephron.

Figures 4,5 (E-J) Distal tubule
Beyond the thin limb of the loop of Henle, the reaction was assigned to the cross sections in the distal tubular portion of the nephron. First to the thick ascending limb as it crosses the inner (E), outer (F) stripes of the outer medulla, and the cortex (G) of the kidney. Past the macula densa the reaction was assigned to cross sections in the distal convoluted tubule (DCT) (H), to connecting segments (CNT) (I) or to the pars arcuata (ACT) (J).



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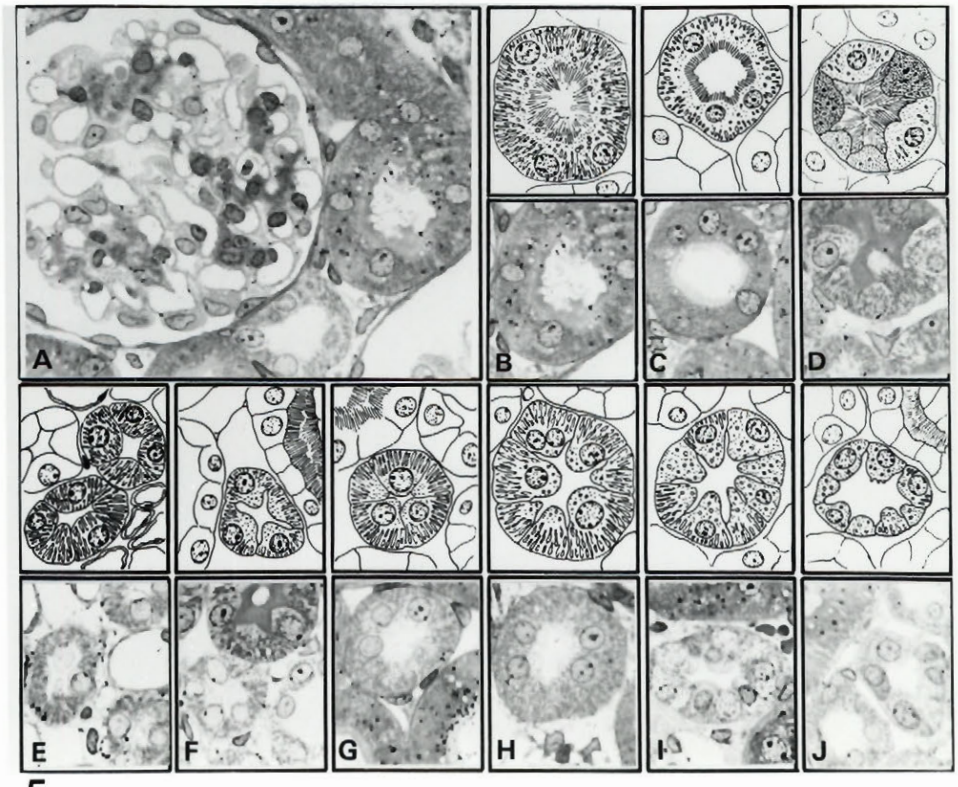

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PART-III

RESULTS

In Vivo Binding of PTH and CT in the Liver Tissue of Normal Rodents

Binding of ^{125}I-bPTH and ^{125}I-sCT in rat liver

In light microscope radioautographs of liver tissue obtained from normal Experimental rats two minutes after intravenous injection of ^{125}I-bPTH(1-34) (Fig. 6), ^{125}I-bPTH(1-84) (Fig. 8), and ^{125}I-sCT (Fig. 10), the silver grains were related to most cells constituting the liver parenchyma. The reaction appeared more intense on those aspects of the liver cords facing sinusoids. In corresponding radioautographs obtained from Control animals (Figs. 7,9,11), the binding of labeled hormone was inhibited by the excess unlabeled related hormone with the remaining reaction being largely associated with the sinusoidal elements of the liver parenchyma.

Statistical analysis of the silver grain counts from radioautographs of liver from the Experimental and the Control animals using the Student's T test indicated a significant decrease in reaction in the tissue of the Control rats ($P<0.001$; Graph 1). Binding of ^{125}I-bPTH was not inhibited, but rather slightly increased, by co-injection with excess unlabeled insulin (Table 5). Similarly, the binding of ^{125}I-sCT was either not affected by co-injection of excess unlabeled insulin, or was increased ($P<0.001$; Table 8).

Binding of ^{125}I-sCT in mouse liver

In normal Experimental mice (Fig. 12), ^{125}I-sCT also bound intensely to the liver parenchyma, and the distribution of the reaction was identical to that observed in the liver of normal Experimental rats (Fig. 12). However, the reaction was not reduced by co-injection of excess unlabeled sCT (Fig. 13; Graph 2).

Statistical analysis of the data indicated a non-significant difference ($P < 0.321$; Graph 2) between the reaction over the tissue obtained from the Experimental animals and that obtained from the Control mice.

Binding of ^{125}I-bPTH and ^{125}I-sCT to hepatocytes and sinusoidal cells in rats

The distribution of the silver grains over hepatocytes, sinusoidal endothelial cells, and Kupffer cells was studied in electron microscope radioautographs. When related to hepatocytes the majority of the silver grains after injection of ^{125}I-bPTH (Fig. 14) and ^{125}I-sCT (Fig. 15) into Experimental animals was related to the plasma membrane. Additionally, some silver grains were seen associated with various ultrastructural elements including those in the Golgi region situated in close proximity to the bile canaliculi. On the sinusoidal endothelial cells (Figs. 16, 17) the silver grains were found over smooth or coated pit formations, on the cell surface plasma membrane or over the membrane of macropinocytotic vacuoles of variable shapes and sizes usually containing fluffy material.

In electron microscope radioautographs obtained from the Control animals co-injected with excess related unlabeled hormone, the reaction over hepatocytes was completely inhibited. The reaction over the sinusoidal endothelial cells appeared decreased, but to a lesser extent.

Binding of ^{125}I-bPTH(1-84) to Kupffer cells in rats

When ^{125}I-bPTH(1-84) was injected in Experimental animals, an intense reaction was observed over Kupffer cell processes (Fig. 18). The reaction was on the cell surface or associated with the membrane of large vacuoles. In Control animals, this reaction was decreased.

In Vivo Binding of CT in Liver tissue of Osteopetrotic Rodents

Based on light microscope radioautography, binding of ^{125}I-sCT was increased significantly in the liver tissue of osteopetrotic op/op and microphthalmic mi/mi mutant mice, when compared with binding in their respective, phenotypically normal siblings ($P<0.001$; Graph 3). No statistically significant difference in binding was observed in osteosclerotic oc/oc, nor in incisor absent ia/ia rodents when compared with the reaction in their phenotypically normal sibling ($P<0.031$; Graph 3).

The statistically significant increase in ^{125}I-sCT binding to liver tissue of op/op mice was paralled by the presence of dense staining cytoplasmic bodies in the Golgi region near the bile canaliculi of hepatocytes and in sinusoidal endothelial cells (Fig. 19). The dense cytoplasmic bodies were also seen in Kupffer cells (Fig. 20).

PLATE 3

Binding of parathyroid hormone and calcitonin in liver tissue of normal rodents. Light microscope radioautography.

Figures 6,7 — Binding of bPTH(1-34) in Experimental (Fig. 6) and in Control (Fig. 7) animals.
BPTH(1-34) Exp 1 Normal rats. Exposure 6 weeks.

Figures 8,9 — Binding of bPTH(1-84) in Experimental (Fig. 8) and Control (Fig. 9) animals.
BPTH(1-84) Exp 6 Normal rats. Exposure 3 weeks.

Figures 10,11 — Binding of sCT in Experimental (Fig. 10) and in Control (Fig. 11) animals.
SCT Exp 7 Normal rats. Exposure 8 weeks.

Figures 12,13 — Binding of sCT in Experimental (Fig. 12) and in Control (Fig. 13) animals.
SCT Exp 3 Normal mice. Exposure 8 weeks.

In rats injected with ^{125}I-bPTH(1-34) (Fig. 6), ^{125}I-bPTH(1-84) (Fig. 8), and ^{125}I-sCT (Fig. 10) the distribution of the radioautographic reaction in liver tissue appears identical. The silver grains are related to most elements of the liver parenchyma. The reaction is more intense on that aspect of the liver cords (LC) facing sinusoids (s). In corresponding radioautographs obtained from the Control animals co-injected with an excess of unlabeled homologous hormone the reaction is greatly decreased. The remaining reaction is largely related to the sinusoidal elements (Figs. 7,9,11).

Although the distribution of ^{125}I-sCT in liver tissue of mice (Fig. 12) is similar to that in rats (Fig. 10), the reaction seen in the Control animal suggests non-competitive binding because the binding of ^{125}I-sCT is not blocked by excess homologous unlabeled hormone. X600.

There was a 30 day interval before the coating of the sections with the radioautographic emulsion.

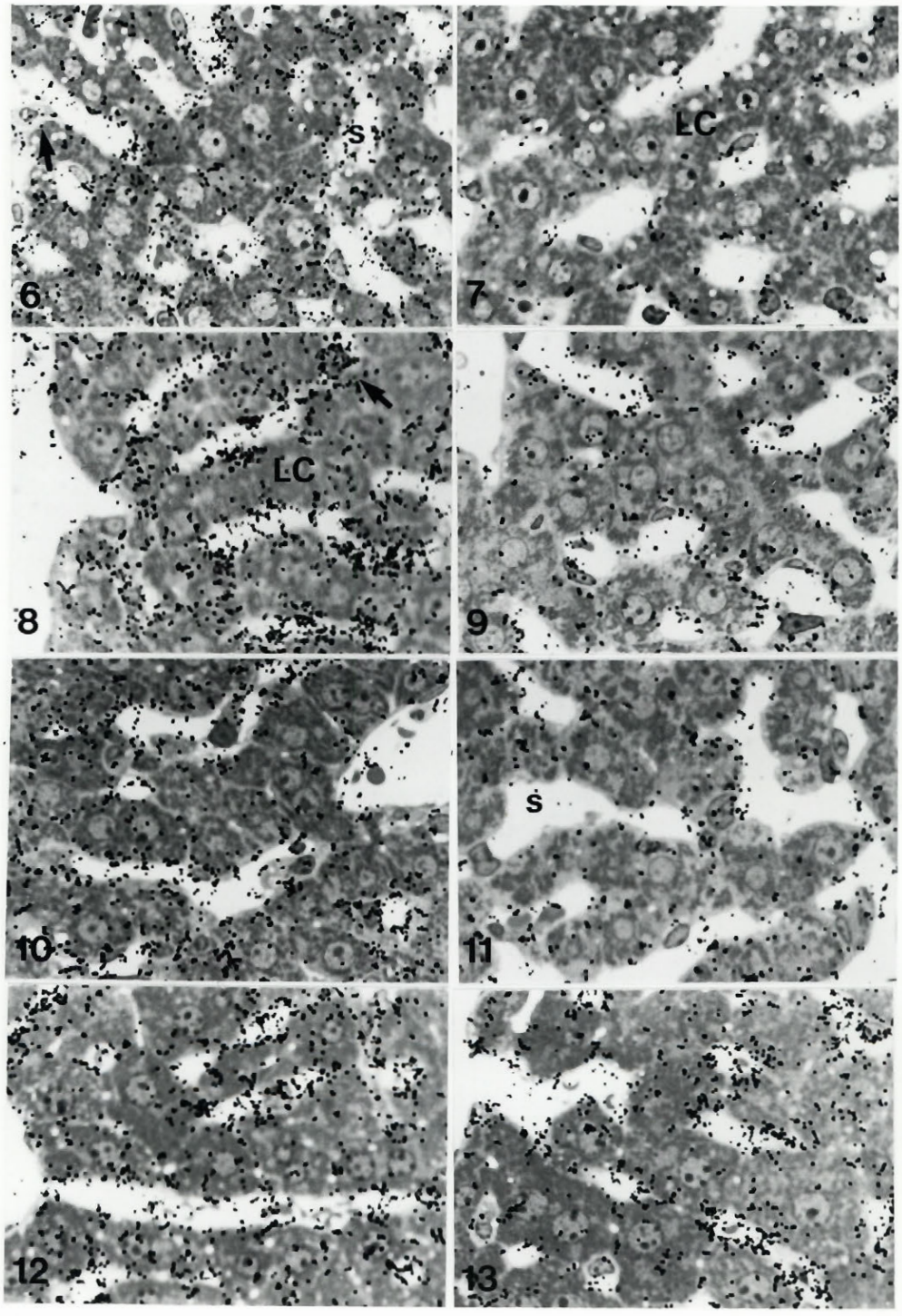

S
6
LC
7
LC
8
9
10
S
11
12
13

PLATE 4

Binding of parathyroid hormone and calcitonin
in liver tissue of normal rodents.
Electron microscope radioautographs.

Figures 14, 16-18 Binding of bPTH(1-84) to hepatocytes (Fig. 14), to sinusoidal cells (Figs. 16,17), and to Kupffer cell processes (Fig. 18).
BPTH(1-84) Exp 6 Normal rat.

Figure 15 Binding of sCT to hepatocytes.
SCT Exp 7 Normal rat.

At the electron microscopic level the filamentous silver grains can be assigned to the different cells of the liver parenchyma. The distribution of the silver grains over hepatocytes (H), and sinusoidal cells (S) is identical in radioautographs of animals injected with ^{125}I-bPTH (Fig. 14) and ^{125}I-sCT (Fig. 15). In hepatocytes the silver grains are found on the plasma membrane (Figs. 14,15; single arrow), over the cytoplasm (double arrow), and related to Golgi elements in close proximity to the bile canaliculus (Fig. 15, insert). This reaction is absent in radioautographs obtained from the Control animal.

Over the sinusoidal cells the silver grains may be found over the smooth aspect of the cell surface or related to coated pits (Fig. 16; single arrow). A great proportion of the grains are related to the membrane of macropinocytotic vacuoles of varying shapes. These contain a small amount of fluffy material (Fig. 17; single arrow). In radioautographs obtained from the Control animals, although decreased to a certain extent, the sinusoidal cell reaction is still present.

The intense reaction associated with the Kuppfer cell processes (K), in animals injected with ^{125}I-bPTH(1-84) is cell-surface related (Fig. 18; single arrow), or associated with the membrane of large vacuoles that also contain fluffy material (Fig. 17; double arrow).

There was a 68 day interval before coating of the sections with the radioautographic emulsion and the duration of exposure was 80 days. X10,375 (Figs. 14, 16-18); X11,375 (Fig. 15).

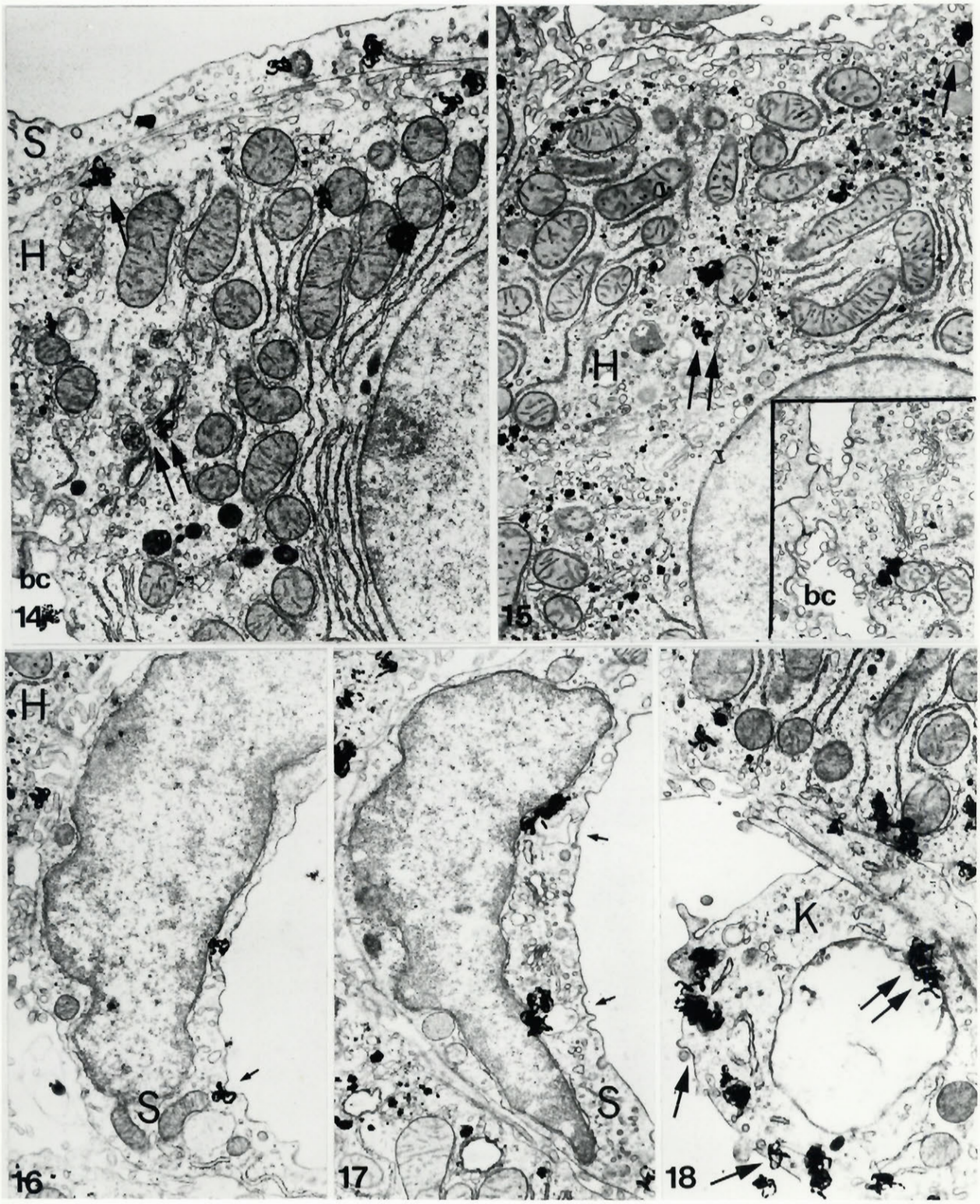
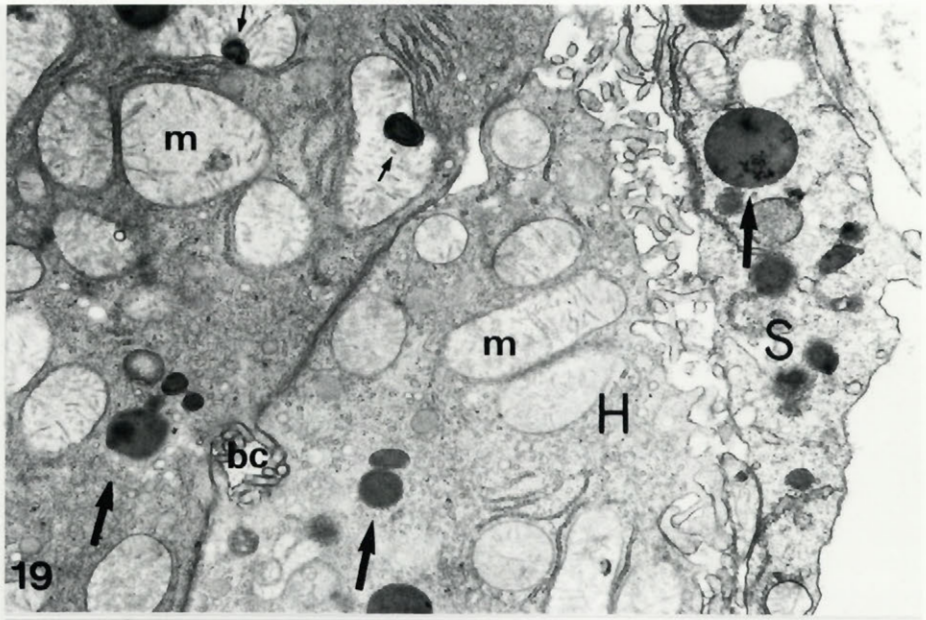

S
H
bc
14
H
15
bc
H
S
16
S
17
K
18

PLATE 5

Electron microscopy of liver tissue from osteopetrotic op/op mice.

Figures 19, 20 Liver tissue of osteopetrotic op/op mice. SCT Exp 1 OP Mutant mice.

The ultrastructure of the hepatic tissue obtained from osteopetrotic op/op mice indicates an increased density of cytoplasmic bodies in hepatocytes (H), and sinusoidal cells (S) (Fig. 19; single arrow) as well as in Kupffer cells (K) (Fig. 20; single arrow). The abnormal dense granules are located in the Golgi area situated in close proximity to the bile canaliculus (Fig.19). In sinusoidal and Kupffer cells the abnormal elements are seen indiscriminately in all portions of the cytoplasm. Moreover, the mitochondria (m) in liver hepatocytes are distended and sometimes contain unusual densities (Fig. 19; small arrow). X13,650; mv, macropinocytotic vacuoles.


m
m
S
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19


K
mv
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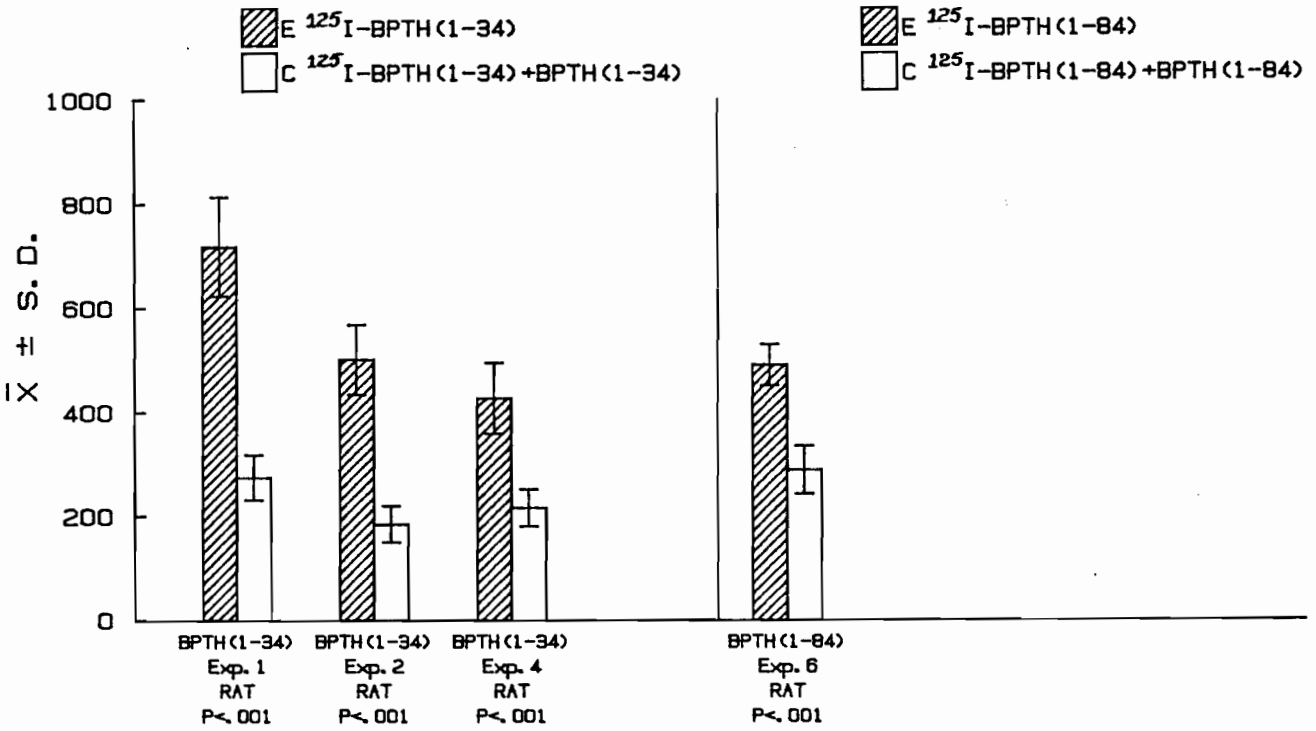
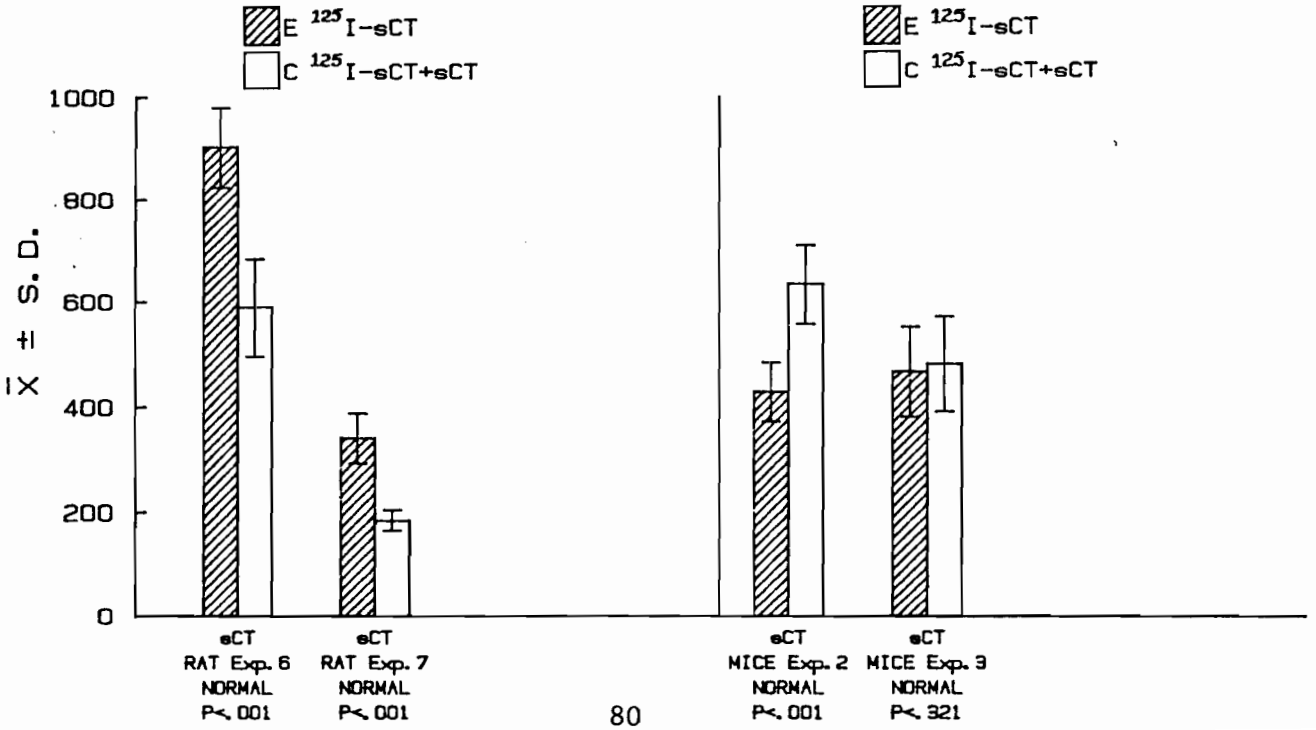
20

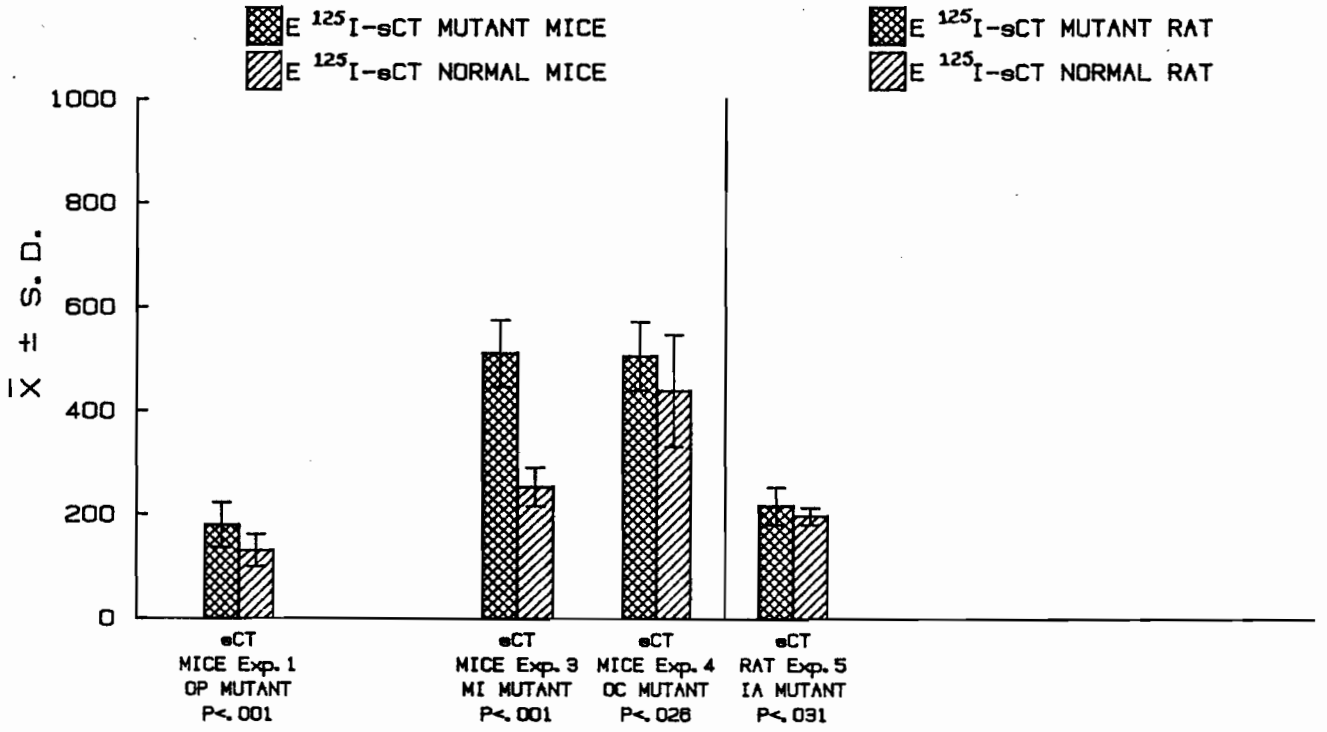
GRAPH 1. Binding of ^{125}I-bPTH in liver tissue of normal rats.



GRAPH 2. Binding of ^{125}I-sCT in the liver tissue of normal rodents.



GRAPH 3. **Binding of ^{125}I-sCT in liver tissue of osteopetrotic mutant rodents.**



In Vivo Binding of PTH and CT in the Skeletal Tissue of Normal Rodents

Light microscope radioautographs of osseous tissue obtained from animals injected with ^{125}I-bPTH alone, showed that much of the radioautographic reaction was related to osteoblasts (Fig. 21). When ^{125}I-sCT was injected the silver grains were located exclusively over osteoclast profiles both in rats (Fig. 23) and in mice (Fig. 24). In corresponding radioautographs obtained from Control animals co-injected with excess unlabeled related hormone the silver grain density was greatly decreased (Figs. 22, 25).

Statistical analysis of the data indicated a significant decrease in the ^{125}I-bPTH reaction associated with osteoblasts in Control animals co-injected with excess unlabeled bPTH ($P<0.001$; 2 experiments out of 3; Graph 4). In the animals injected with ^{125}I-bPTH, the reaction associated with osteoclasts was low and no statistically significant decrease in reaction was seen when animals were co-injected with excess unlabeled bPTH ($P<0.150$; Graph 5).

In animals injected with ^{125}I-sCT alone, the reaction related to osteoblasts was low in both rats and mice, whereas the reaction over osteoclast profiles was high.

Statistical analysis of the silver grain counts revealed no significant decrease in reaction in Control animals injected with unlabeled sCT ($P<0.210$; Graph 6). The reaction over osteoclast profiles was high and significantly decreased by co-injection of excess unlabeled sCT ($P<0.001$; Graph 7) but not by excess unlabeled bPTH ($P<0.276$; Table 6; $P<0.076$; Table 7) nor by excess unlabeled insulin ($P<0.134$; Table 8).

Distribution of ^{125}I-bPTH binding over osteoblasts, sinusoidal and mononuclear connective tissue cells

In electron microscope radioautographs prepared from animals injected with ^{125}I-bPTH alone, two minutes after the intravenous injection the majority of the silver grains were found on the plasma membrane (Fig. 26) or associated with dense bodies closely related to the cell surface of osteoblasts (Figs. 27, 28). In some cases, the silver grains were arranged around what appeared to be Golgi areas (Fig. 29). In electron microscope preparations obtained from Control animals this reaction was totally inhibited by co-injection of excess related unlabeled hormone.

Additionally, silver grains were observed in relation to sinusoidal endothelial cells and to mononuclear connective tissue cells. The silver grains over the sinusoidal cells (Figs. 30-32) were associated with the cell surface and with light, as well as electron dense cytoplasmic bodies. When related to the mononuclear connective tissue cell (Figs. 33,34), recognized by its single large irregular nucleus and by numerous Golgi stacks, the silver grains were over the cell surface and related to elements in the Golgi area, or to dense cytoplasmic bodies in proximity to the nucleus.

Distribution of the ^{125}I-sCT binding over osteoclasts

In electron microscope radioautographs prepared from animals injected with ^{125}I-sCT the majority of silver grains were found over most of the cell surface of the osteoclast profiles except for the ruffled border (Figs. 35-38). In mice, the reaction was distributed similarly (Figs. 39, 40). Electron microscope radioauto-

graphs obtained from Control animals showed that the reaction was completely inhibited by co-injection of unlabeled sCT.

In Vivo Binding of CT in Skeletal Tissue of Osteopetrotic Rodents

Osteopetrotic and microphthalmic mice

To study the binding of labeled hormone in osteopetrotic mutant rodents and in their phenotypically normal littermates, the same quantity of ^{125}I-sCT was injected into two sets of animals. The radioautographic reaction related to osteoclast profiles appeared to be increased in osteopetrotic op/op mice (Figs. 41,42) and in microphthalmic mi/mi mice (Figs. 45,46) when compared with the reaction in their respective phenotypically **normal** osteopetrotic (Figs. 43,44) and microphthalmic (Figs. 47,48) littermates.

Quantitation and statistical analysis of the radioautographic reaction indicated a significant increase in binding over osteoclast profiles in op/op mutant mice (Experiments 1 and 2; $P<0.001$; Graph 8) and in mi/mi mutant mice (Experiment 3; $P<0.001$; Graph 8).

Osteosclerotic and incisor absent rodents

In light microscope radioautographs obtained from osteosclerotic oc/oc mutant mice (Fig. 58) and from incisor absent ia/ia rats (Fig. 60) injected with ^{125}I-sCT, no reaction was seen over osteoclast profiles. The expected osteoclast reaction was present in the osteosclerotic, but phenotypically normal littermate (Fig. 59).

Statistical analysis of the quantitative data indicated a significant decrease in radioautographic reaction related to osteoclasts in the tissue of osteosclerotic oc/oc mice when compared

to the reaction in its normal sibling (Experiment 4; $P<0.001$; Graph 8). The reaction was absent in radioautographs obtained from the incisor absent ia/ia mutant rats as well as from its presumably phenotypically normal littermate (Experiment 5; Graph 8).

In electron microscope radioautographs obtained from osteopetrotic op/op mutant mice the increase in reaction was paralleled by an increase in cell surface as the cell membrane was thrown into numerous evaginations (Figs. 50-52). These often formed bleb-like outward projections (Figs. 51-52). An abnormally large number of light and dense cytoplasmic bodies were observed in the cell cytoplasm close to the cell surface away from the bone (Figs. 51,52).

In the same animals, osteoblasts appeared abnormally active with distended Golgi areas and large mitochondria (Fig. 53). Numerous conspicuous, abnormal cytoplasmic bodies were also observed in the cytoplasm of the osteoblasts in this mutant (Fig. 54).

In electron microscope radioautographs obtained from microphthalmic mi/mi mice, the increase in radioautographic reaction was distributed over a rather smooth cell surface (Fig. 56). The distribution suggested a localized increase in ^{125}I-sCT binding (Fig. 57).

PLATE 6

Binding of parathyroid hormone and calcitonin in osseous tissue of normal rodents. Light microscope radioautography

Figures 21,22 Binding of bPTH(1-84) in Experimental (Fig. 21) and in Control (Fig. 22) animals.
BPTH(1-84) Exp 6 Normal rat. Exposure 3 weeks.

Figure 23 Binding of sCT in Experimental animal.
SCT Exp 7 Normal rat. Exposure 1 week.

Figures 24,25 Binding of sCT in Experimental (Fig. 24) and in Control (Fig. 25) animals.
SCT Exp 1 Normal mice. Exposure 6 weeks.

In animals injected with ^{125}I-bPTH(1-34) and ^{125}I-bPTH(1-84), the silver grains are associated with all osteoblasts (ob), including those aligned on the mixed spicules in the tibial proximal metaphysis (Fig. 21). Some grains are related to bone connective tissue. Two minutes after intravenous injection, the silver grains are found over the cell surface and the pale Golgi area of the osteoblasts (Fig. 21; arrow). In corresponding radioautographs obtained from the Control animal co-injected with an excess of unlabeled homologous hormone, the silver grains density is decreased (Fig. 22). ^{125}I-bPTH binding was not observed over osteoclasts.

^{125}I-sCT binds to osteoclast profiles in rats (Fig. 23; arrow), as well as in mice (Fig. 24; arrow). The reaction is blocked in tissue obtained from the Control animals co-injected with an excess of unlabeled calcitonin (Fig. 25; arrow).

There was a 47 day interval before coating of the sections with the radioautographic emulsion. X600; s, sinusoid; ms, mixed spicule.

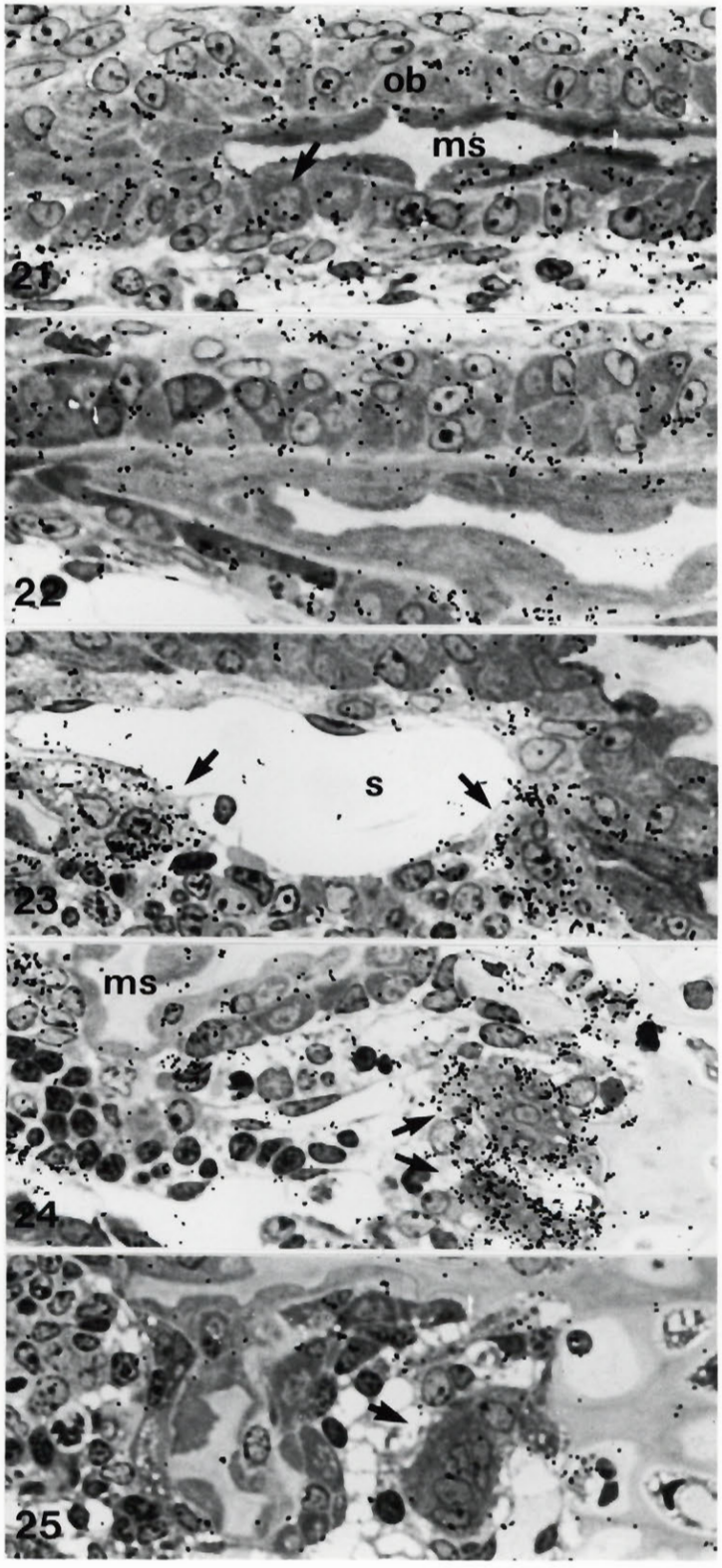

ob
ms
21
22
s
23
ms
24
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PLATE 7

Binding of parathyroid hormone to osteoblasts.
Electron microscope radioautography.

Figures 26-29 Binding of bPTH(1-84) in Experimental animal.
BPTH(1-84) Exp 6 Normal rat. Exposure 103 days.

Two minutes after the intravenous injection of ^{125}I-bPTH(1-34) or ^{125}I-bPTH(1-84) the silver grains are localized on the surface of osteoblasts (Figs. 26, 27; single arrow) or associated with dense bodies closely related to the cell surface (Figs. 27,28; double arrows). In some of the osteoblast profiles, the filamentous silver grains are arranged around what appears as a pale Golgi area in light microscope histological section (Fig. 21; double arrow) and what appears as a dense area in the electron micrograph (Fig. 29; area delineated by arrows). In electron microscope radioautographs obtained from the Control animal this reaction was maximally blocked by co-injection of unlabeled homologous hormone.

There was an 85 day interval before the coating of the sections with the radioautographic emulsion.
X8,250 (Fig. 26), X12,500 (Figs. 27, 29), and X20,000 (Fig. 27). B, bone.

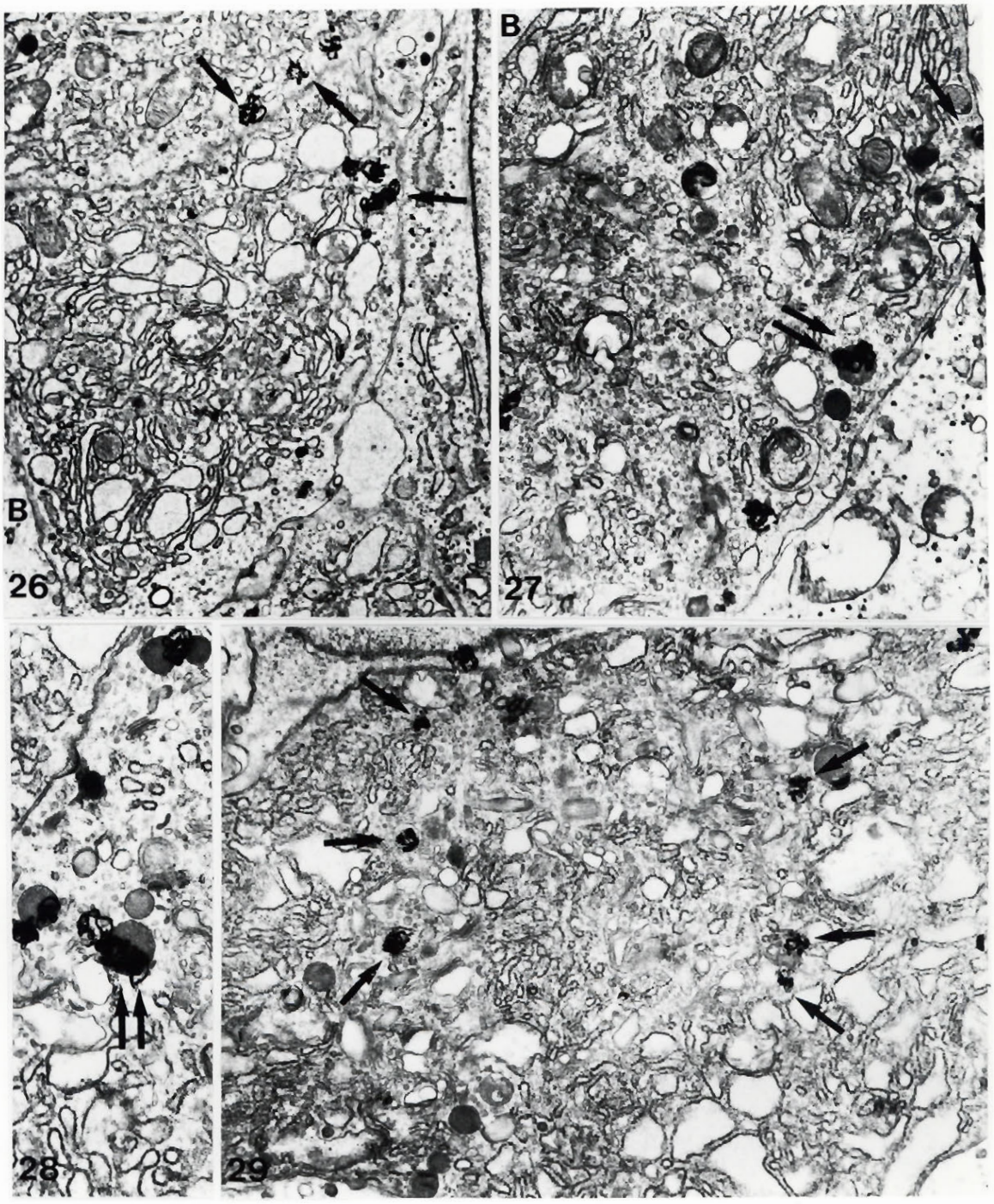

B
26
B
27
28
29

PLATE 8

Binding of parathyroid hormone
to sinusoidal and mononuclear bone cells.
Electron microscope radioautography.

Figures 30-32 Binding of bPTH(1-84) to sinusoidal cells of osseous tissue.
BPTH(1-84) Exp 6 Normal rats. Exposure 103 days.

Figures 33,34 Binding of bPTH(1-84) to a bone connective tissue cell.
BPTH(1-84) Exp 6 Normal rat. Exposure 103 days.

In animals injected with ^{125}I-bPTH(1-34) and ^{125}I-bPTH(1-84), beside the competitive binding related to osteoblasts, binding occurs to bone sinusoidal cells (S) (Fig. 30; single arrow). The silver grains may be found on the cell surface (Fig 30; arrow), or related to light and dense cytoplasmic bodies (cb) (Figs. 31, 32).

The filamentous grains are related to a mononuclear bone connective tissue cell as well. The cell is characterized by its single irregular nucleus, and by its profusion of Golgi stack elements. The silver grains may be found on the cell surface (Fig. 33; single arrow), related to Golgi elements (double arrow), or to dense bodies in close proximity to the nucleus (Fig. 34).

There was an 85 day interval before the coating of the sections with the radioautographic emulsion. X12,500. N, nucleus.

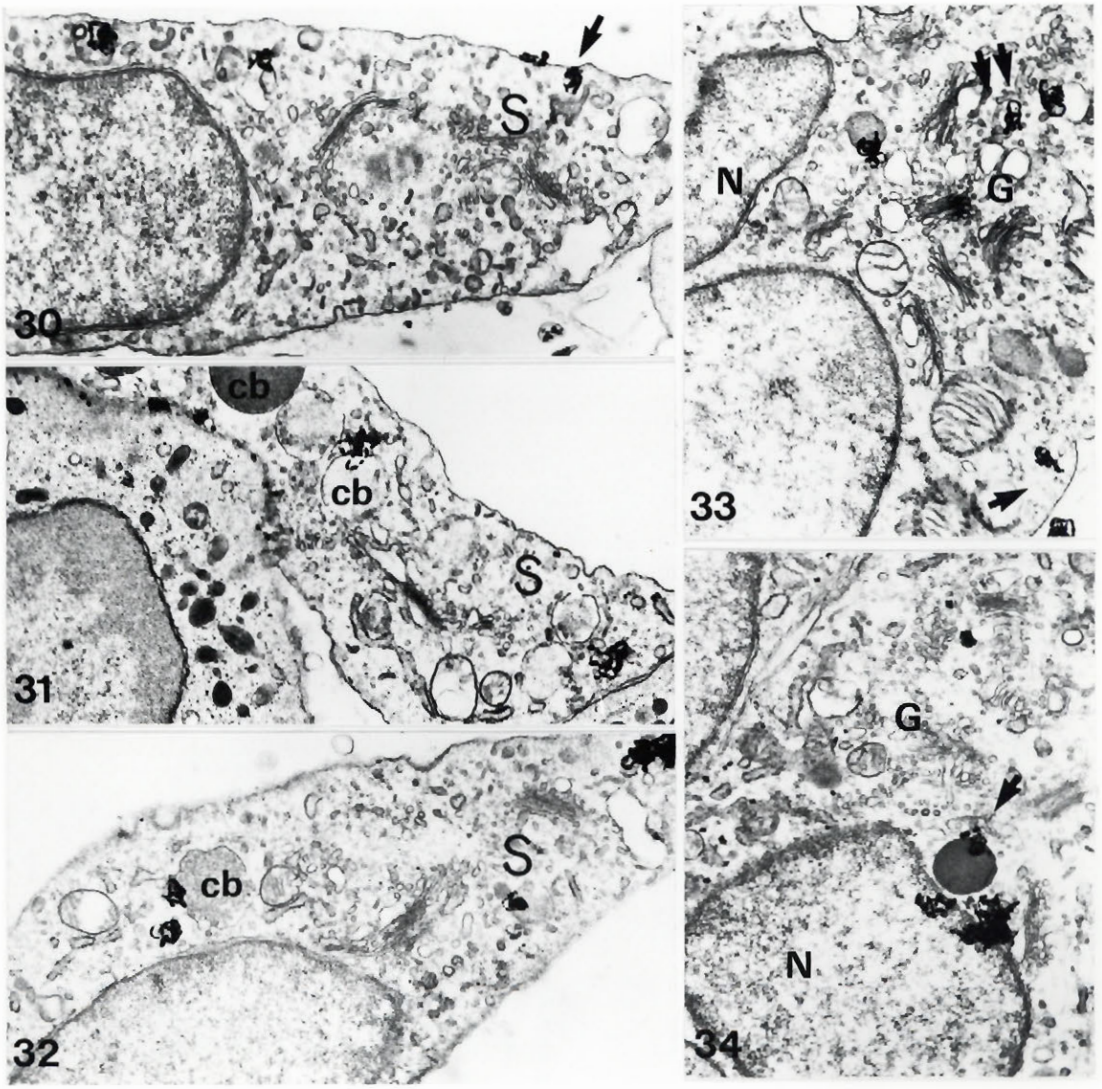

S
30
cb
cb
S
31
cb
S
32
N
G
33
G
N
34

PLATE 9

Binding of salmon calcitonin to osteoclasts
in normal rats.
Electron microscope radioautography.

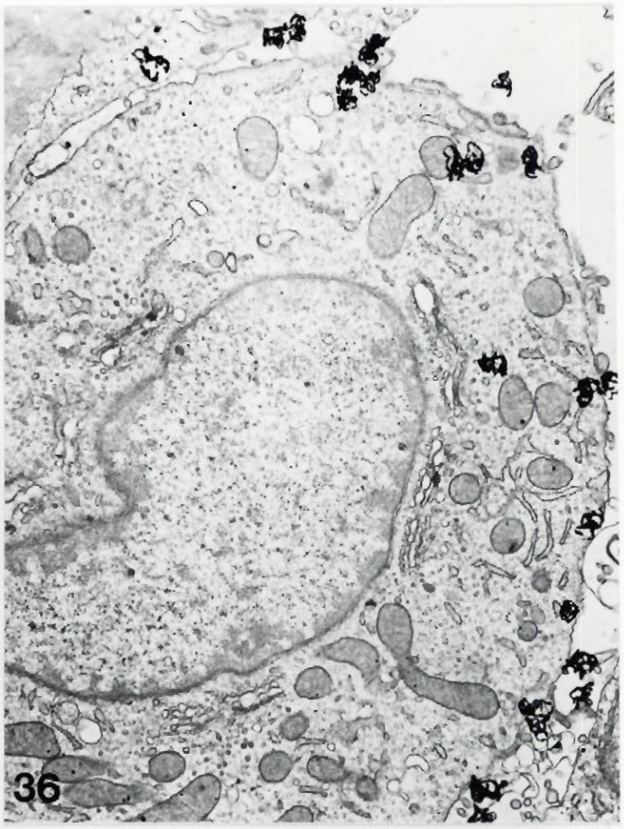
Figures 35-38 Binding of iodinated calcitonin to osteoclasts.
SCT Exp 7 Normal rat. Exposure 80 days.

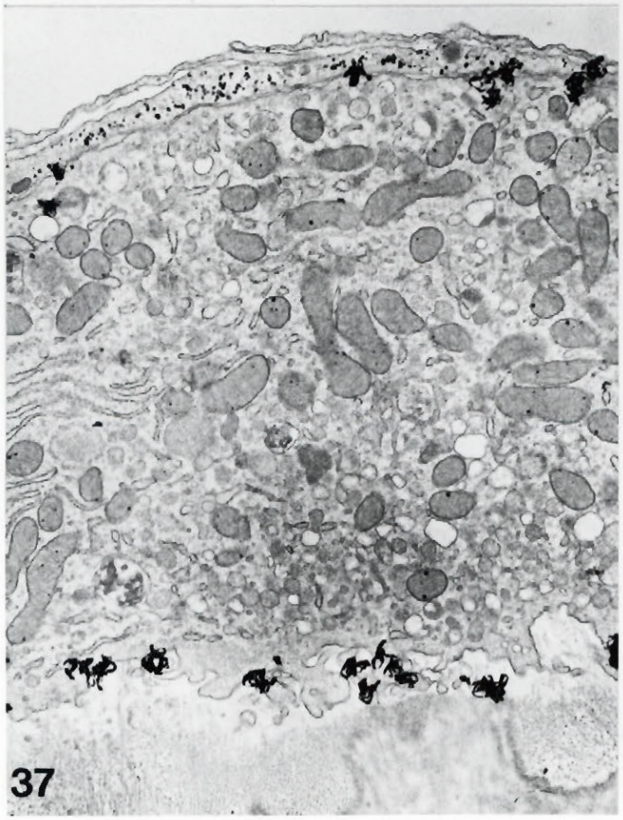
The osteoclast was first described by Kolliker in 1873. Although mononuclear osteoclasts have been reported, osteoclasts are best known as large multinucleated cells. They are often polarized with one or more sides of the cell arranged in ruffled border. This specialization of the cell surface is directly apposed to calcified matrix, and is surrounded by a clear zone (cz). This zone consists of a fine amorphous granular material devoid of any cell organelles. Immediately above the ruffled border is the zone of vesicles and vacuoles. The cytoplasm, typical of osteoclasts, carries an abundance of mitochondria and rough endoplasmic reticulum. The Golgi is made up of groups of flattened saccules placed at interval around the nucleus. It is associated with a variety of small and large cytoplasmic bodies.

In osseous tissue ^{125}I-sCT binds to osteoclast profiles. The silver grains are located on the free surface (Figs. 35-37) up to the ruffled border area which suggests an absence of receptors over this region of the cell membrane (Fig. 38).

There was an 86 day interval before the coating of the sections with the radioautographic emulsion. X8,550. B, bone; C,cartilage.


C
B
35


36


37

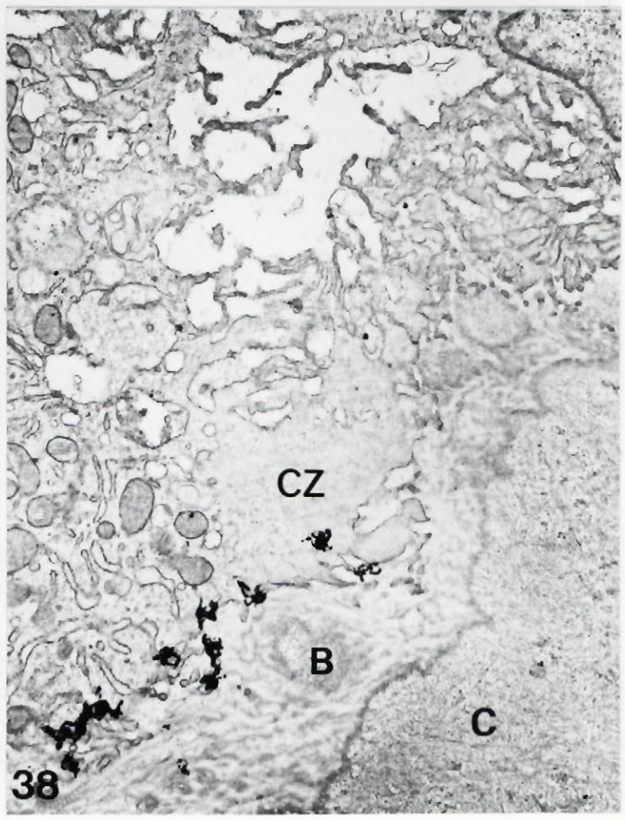

CZ
B
C
38

PLATE 10

Binding of salmon calcitonin to osteoclasts
in normal mice
Electron microscope radioautography.

Figures 39,40 Binding of salmon calcitonin to osteoclasts.
SCT Exp 3 Normal mice. Exposure 80 days.

In mature mice the osteoclasts are often found away from the bone surface (Fig. 39). In many cases they are associated with the bone matrix (Fig. 40), or separated from the bone by flattened cells characterized by the virtual absence of cytoplasmic organelles and formed mainly by the nucleus surrounded with a thin rim of cytoplasm.

The cell membrane in direct contact with the bone is very seldom arranged in a well developed ruffled border; it resembles what has been called a transitional or clear zone (cz). The nucleus often takes an unsual shape, and the Golgi elements are often found in clusters (Fig. 39; single arrow) rather than arranged in groups of flattened sacs placed at intervals around each nucleus. The absence of a ruffled border, a zone of vesicles and vacuoles, as well as the sparcity of cytoplasmic bodies suggest that in the mature mice, the osteoclasts are inactive.

Two minutes after the intravenous injection the silver grains representing the binding of ^{125}I-sCT are numerous and located over all of the osteoclast cell surface with the exception of the ruffled border (Figs. 39, 40). This reaction is totally inhibited by excess homologous unlabeled hormone.

There was an 86 day interval before the coating of the sections with the radioautographic emulsion. X6,750 (Fig. 39); X8,550 (Fig. 40). B, bone.

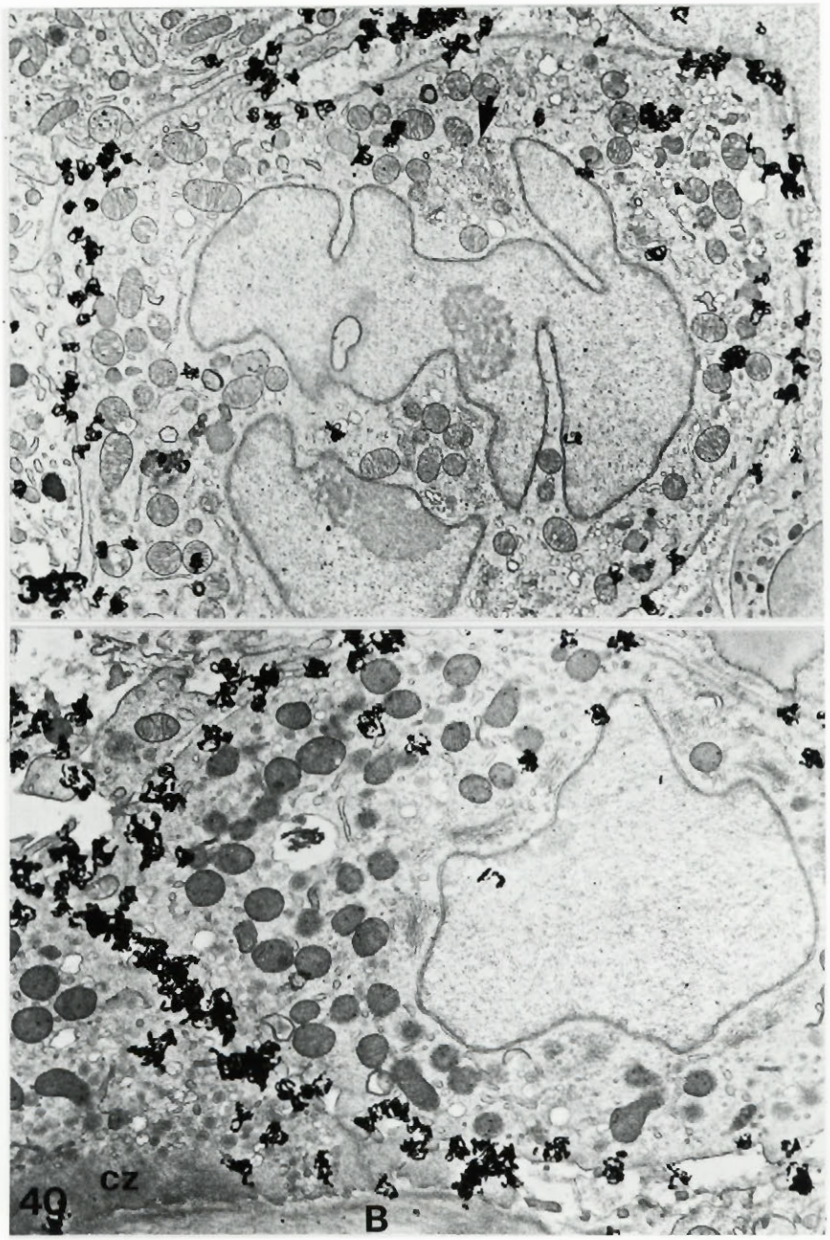

CZ
B

PLATE 11

Increased calcitonin binding to osteoclasts
in osteopetrotic op/op and microphthalmic mi/mi mice
Light microscope radioautography.

Figures 41-44 Binding of ^{125}I-sCT in osteopetrotic op/op mice (Figs. 41,42) and in the phenotypically normal littermates (Figs. 43,44).
SCT Exp 2 OP Mutant mice. Exposure 6 weeks.

Figures 45-48 Binding of ^{125}I-sCT in microphthalmic mi/mi mice (Figs 45,46) and in the phenotypically normal littermates (Figs 47,48)
SCT Exp 3 Mi Mutant mice. Exposure 3 weeks.

In osseous tissue calcitonin binds exclusively to osteoclast profiles. Osteopetrosis is a bone disease characterized by skeletal sclerosis and abnormal bone resorption due presumably to defective osteoclasts.

Binding of ^{125}I-sCT in osteopetrotic mutants was compared with binding in their respective phenotypically normal siblings.

In op/op (Figs. 41,42) and mi/mi (Figs. 45,46) osteopetrotic mutants, there is an increase in calcitonin binding when compared to the binding in their phenotypically normal osteopetrotic (Figs. 43,44) and microphthalmic (Figs. 47,48) littermates.

In osteopetrotic op/op mice, the osteoclasts are structurally abnormal. They are seldom multinucleated and have a high cytoplasm-nuclear ratio (Figs. 41,42). In microphthalmic mi/mi mice, although more numerous, the cells are smaller with a low cytoplasm-nuclear ratio (Figs. 45,46).

There was a 45 day interval before the coating of the sections with the radioautographic emulsion. X600. ms, mixed spicule; s, sinusoid.

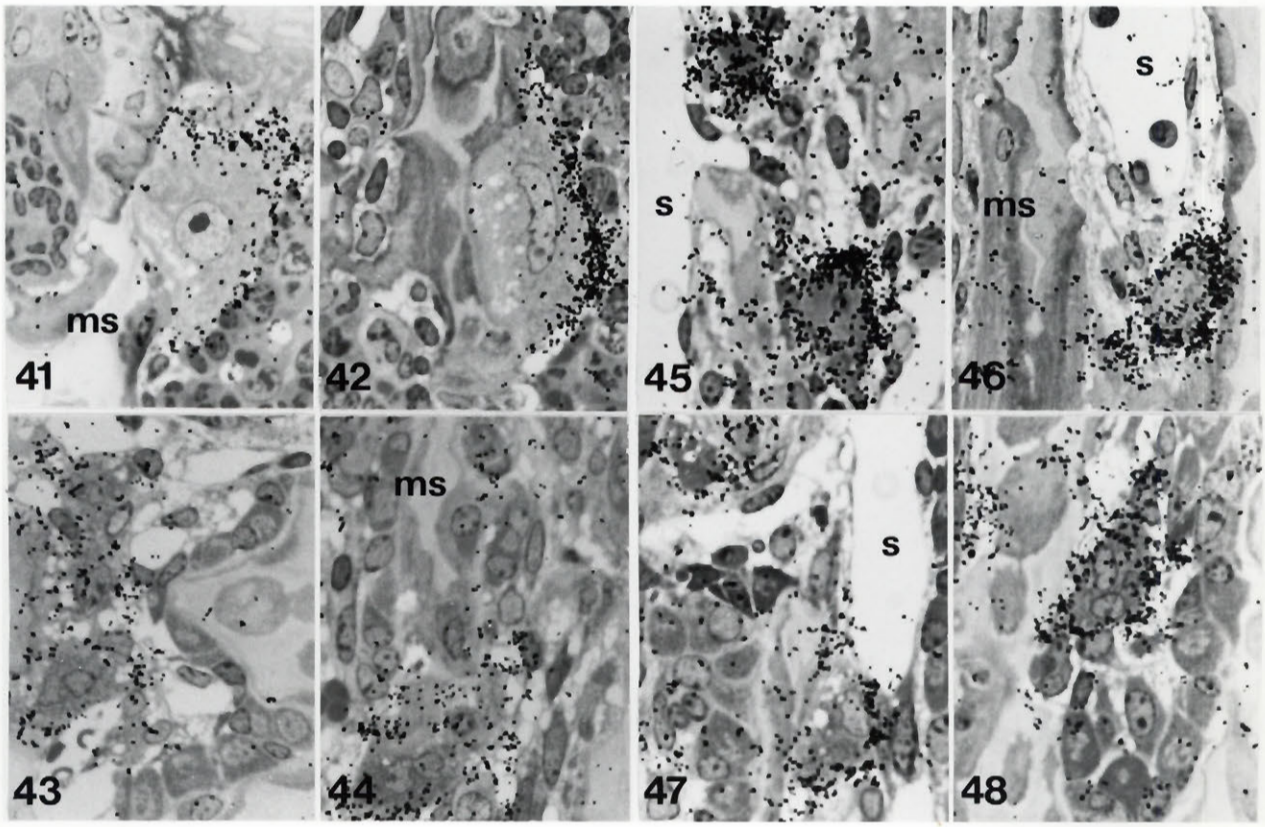

ms
41
42
s
45
s
ms
46
43
ms
44
s
47
48

PLATE 12

Increase in calcitonin binding to osteoclasts
in osteopetrotic op/op mutant.
Electron microscope radioautography.

Figures 49-52 Binding to osteoclasts in osteopetrotic op/op mutant mice.
SCT Exp 1 OP Mutant mice. Exposure 80 days.

In osteopetrotic animals the osteoclast profiles are smaller, often containing a single nucleus (Fig. 49). The cells may be large and in some cases, the surface is thrown into complicated and elaborate cell surface projections (Fig. 50). The surface apposed to bone or calcified cartilage often fails to form a normal ruffled border (Fig. 49; arrows).

Binding of ^{125}I-sCT to osteoclasts in op/op mutant mice is significantly increased when compared with the binding in their phenotypically normal littermates. The increase in binding is accompanied by an increase in cell surface membrane (Figs. 51,52; arrow). The cytoplasm of the cell away from the bone surface contains a variety of dense cytoplasmic bodies (cb).

There was an 80 day interval before the coating of the sections with the radioautographic emulsion.
X3,750 (Fig. 49); X8,550 (Fig. 50); X15,000 (Figs. 51,52). N, nucleus.

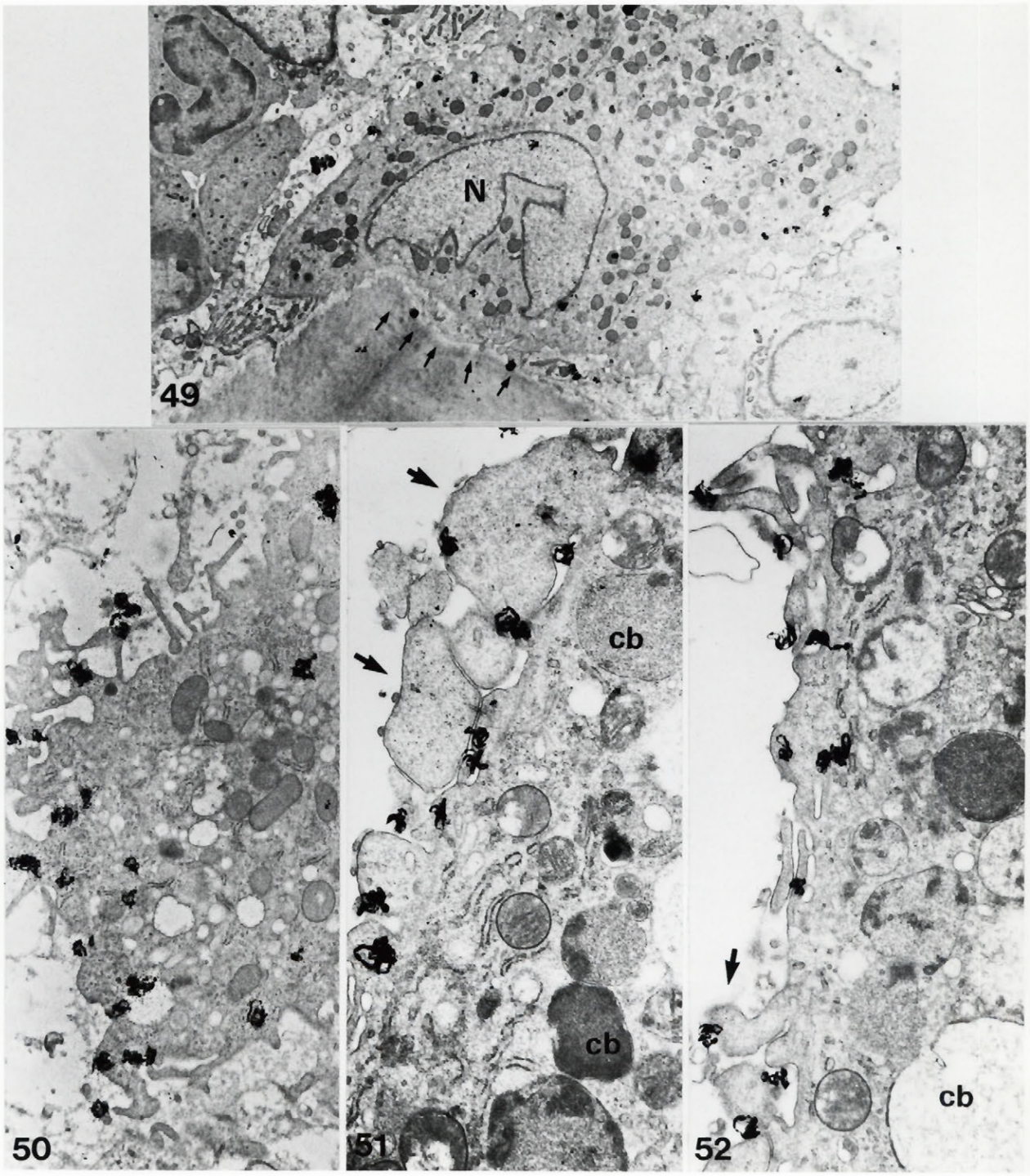

N
49
50
cb
cb
51
cb
52

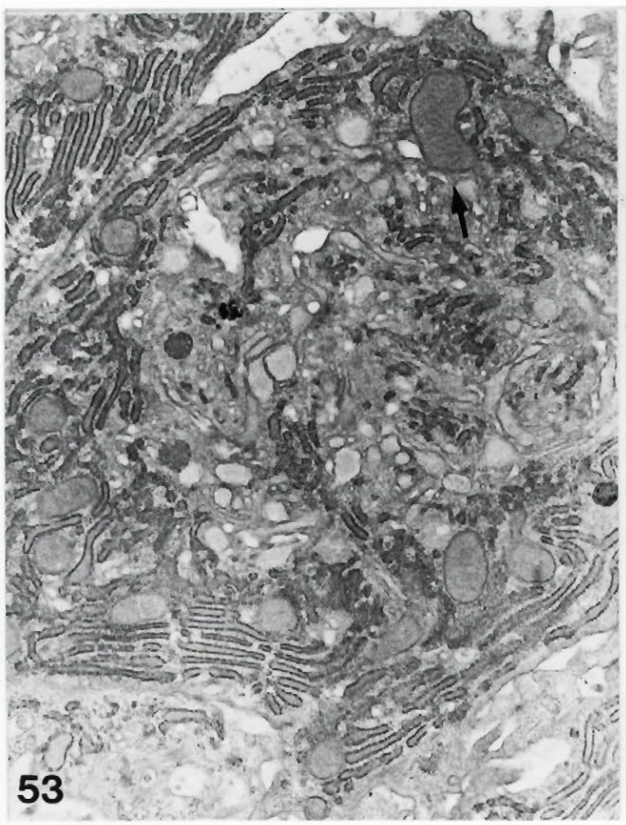
PLATE 13

Possible abnormal osteoblastic structure
in osteopetrotic op/op mutant mice.
Electron microscope radioautography.

Figures 53-54 Illustration of abnormal osteoblastic structure SCT Exp 1 OP Mutant mice.

In osteopetrotic op/op mice, osteoblasts have prominent Golgi areas and often show abnormally shaped mitochondria (Fig. 53; arrow). Some of the osteoblast profiles contain cytoplasmic inclusions identical to those described by Marks (1982) in a study on osteoclasts in osteopetrotic op/op mice (Fig. 54; arrow). These are made of a lipid droplet crowned by a crescent-shaped dense structure and may be indicative of intracellular disposal of non-usable material.

There was an 80 day interval before the coating of the sections with the radioautographic emulsion. X10,650. B, bone.


53


B
54

PLATE 14

Increase in calcitonin binding to osteoclasts in microphthalmic mi/mi mutant mice. Electron microscope radioautography.

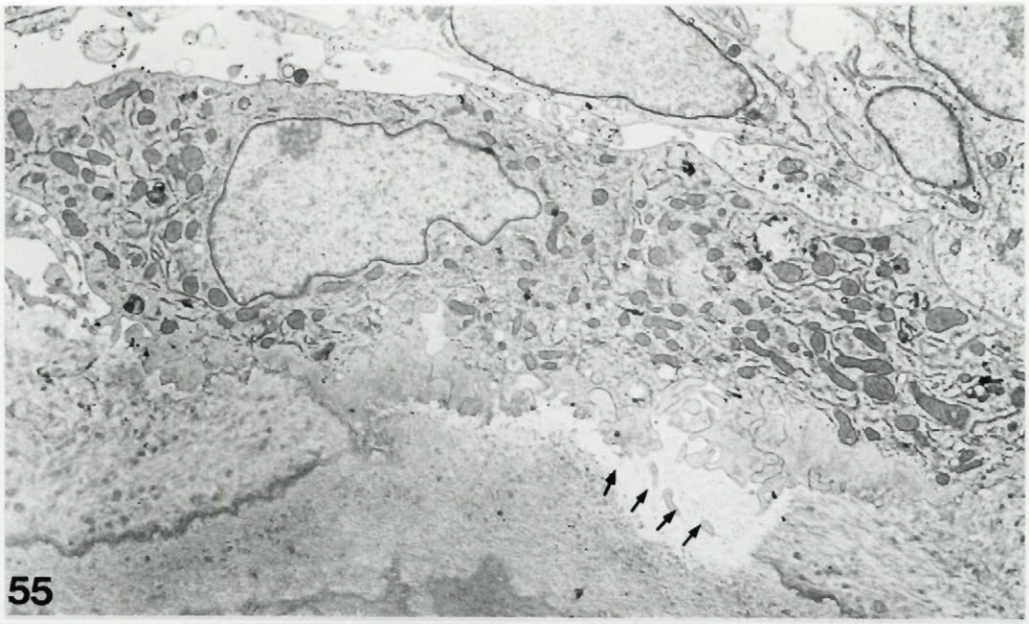
Figures 55-57 Binding of ^{125}I-sCT in microphthalmic mi/mi mice. SCT Exp 3 MI Mutant mice. Exposure time 80 days.

In microphthalmic mi/mi mice, the osteoclasts profiles are smaller. The ruffled border is absent, or when present, it is underdeveloped (Fig. 55; arrows). The cytoplasm is poorly differentiated as illustrated by the sparcity of the cytoplasmic bodies and vacuoles (Holtrop et al., 81).

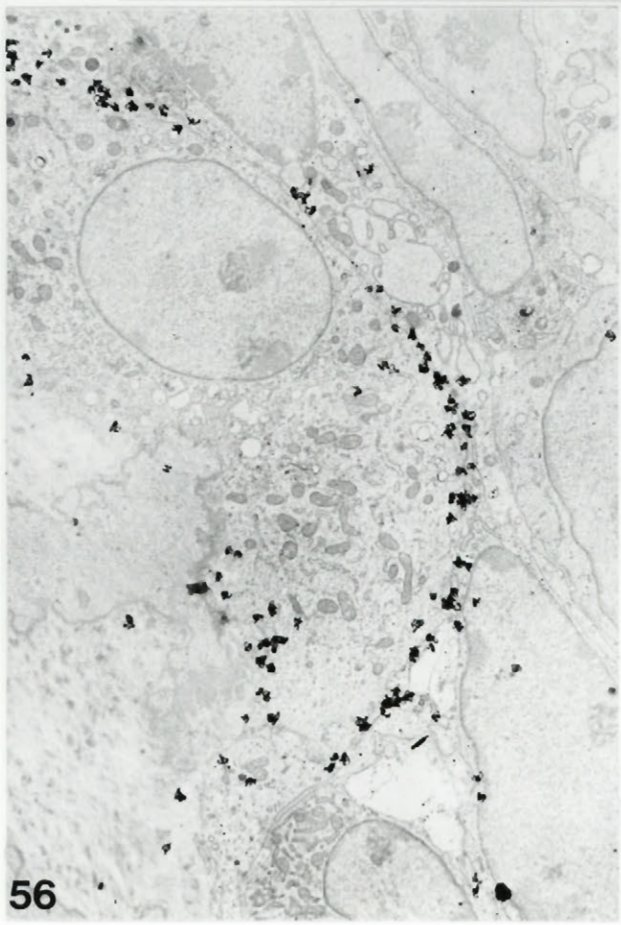
There is a significant increase in ^{125}I-sCT binding to osteoclast profiles in the microphthalmic mi/mi mice when compared to the binding over profiles in the phenotypically normal littermates. The intense radioautographic reaction appears to be evenly distributed over most of the cell surface (Fig. 56), but closer examination suggests clustering of receptors in given areas on the cell surface (Fig. 57; arrows).

There was a 58 day interval before the coating of the sections with radioautographic emulsion.

X5,000 (Fig. 55); X3,125 (Fig. 56); X8,550 (Fig. 57).



55



56

57

PLATE 15

Decreased salmon calcitonin binding to osteoclasts in osteosclerotic oc/oc mice and in incisor absent ia/ia rats. Light microscope radioautography

Figures 58-59 Binding of ^{125}I-sCT in osteosclerotic os/os mice (Fig. 58) and in its phenotypically normal littermate (Fig. 59)
SCT Exp 4 OS Mutant mice. Exposure 3 weeks.

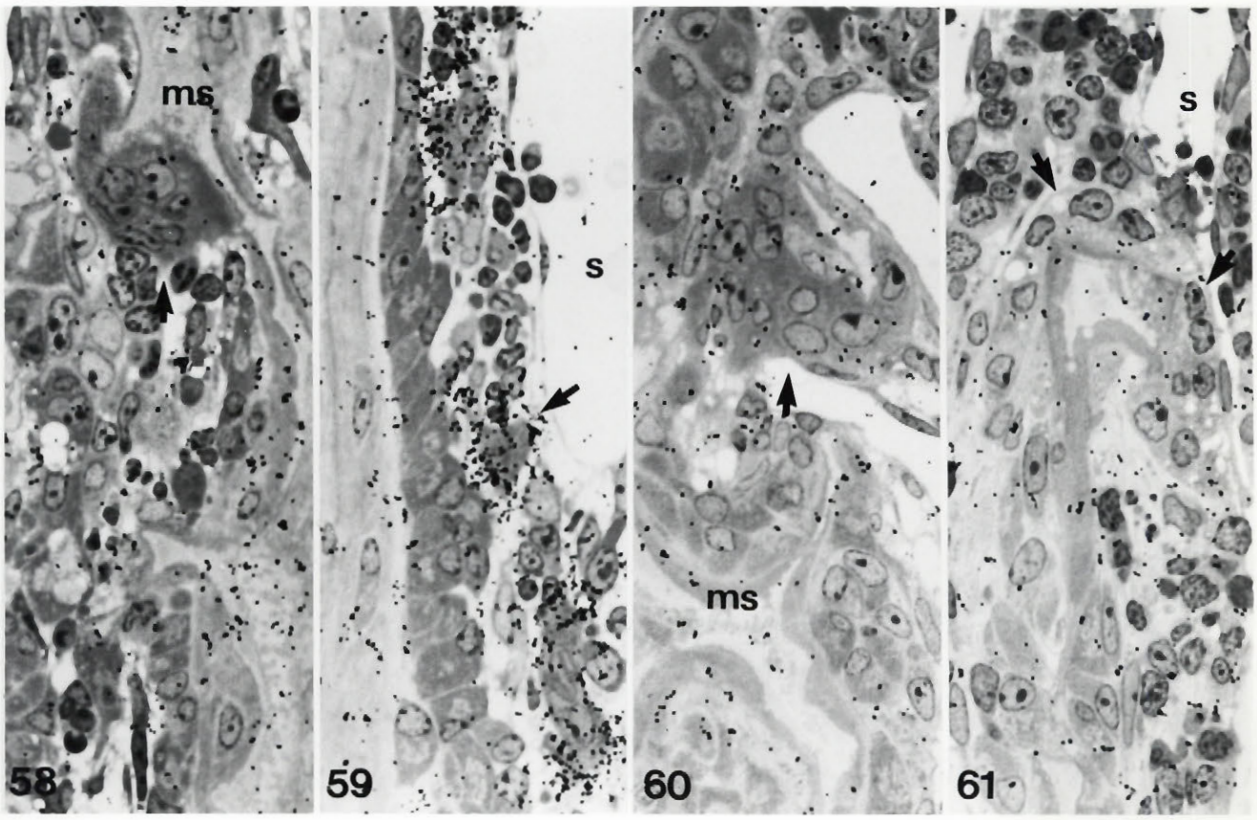
Figures 60-61 Binding of ^{125}I-sCT in incisor absent ia/ia rats (Fig. 60) and in its phenotypically normal littermate (Fig. 61).
SCT Exp 5 IA Mutant rat. Exposure 3 weeks.

In osteosclerotic oc/oc mice, as well as in the incisor absent ia/ia rats, the osteoclast profiles are very large. Their nuclei have prominent nucleoli closely related to the nuclear envelope.

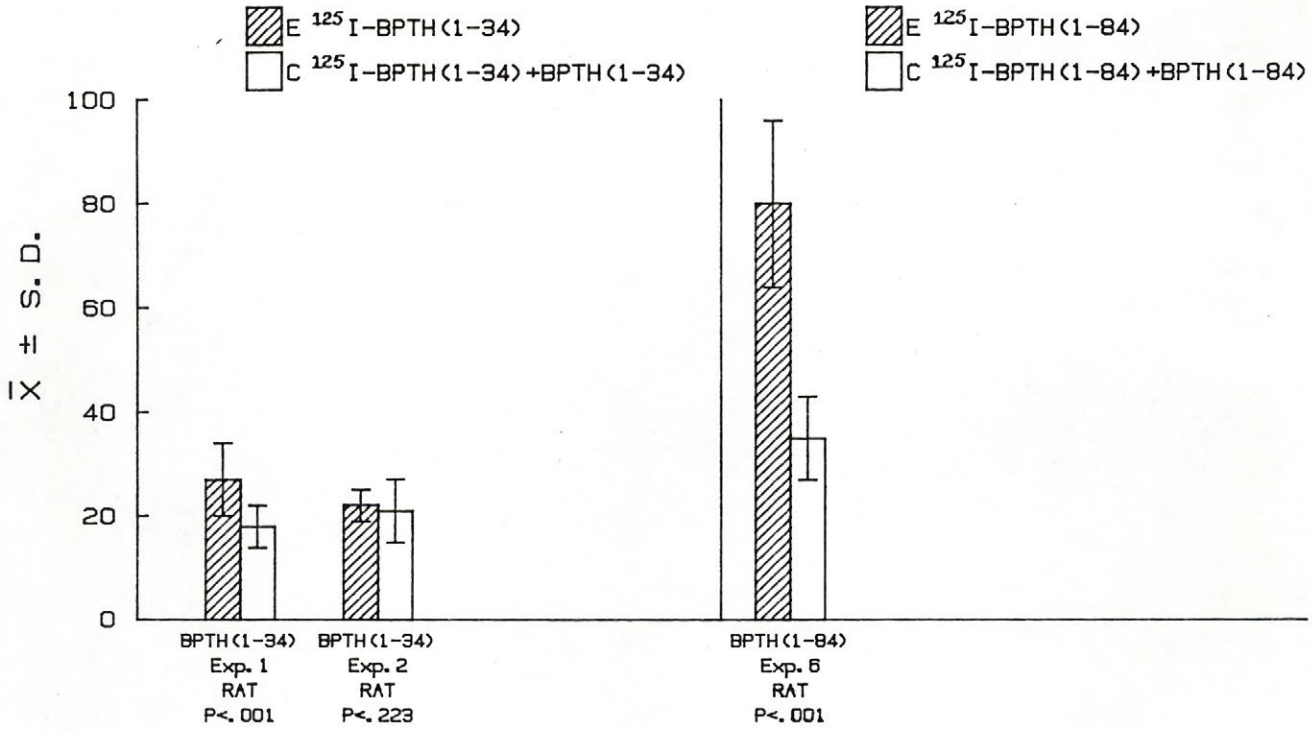
In radioautographs prepared from the osseous tissue of oc/oc (Fig. 58; arrow), and ia/ia rats (Fig. 60; arrow), the exclusive radioautographic reaction associated with osteoclast profiles is absent, but present in the radioautographs prepared from the tissue of the phenotypically normal osteosclerotic littermates (Fig. 59; arrow).

The expected reaction was not observed over osteoclast profiles in radioautographs obtained from the phenotypically normal incisor absent littermates (Fig. 61).

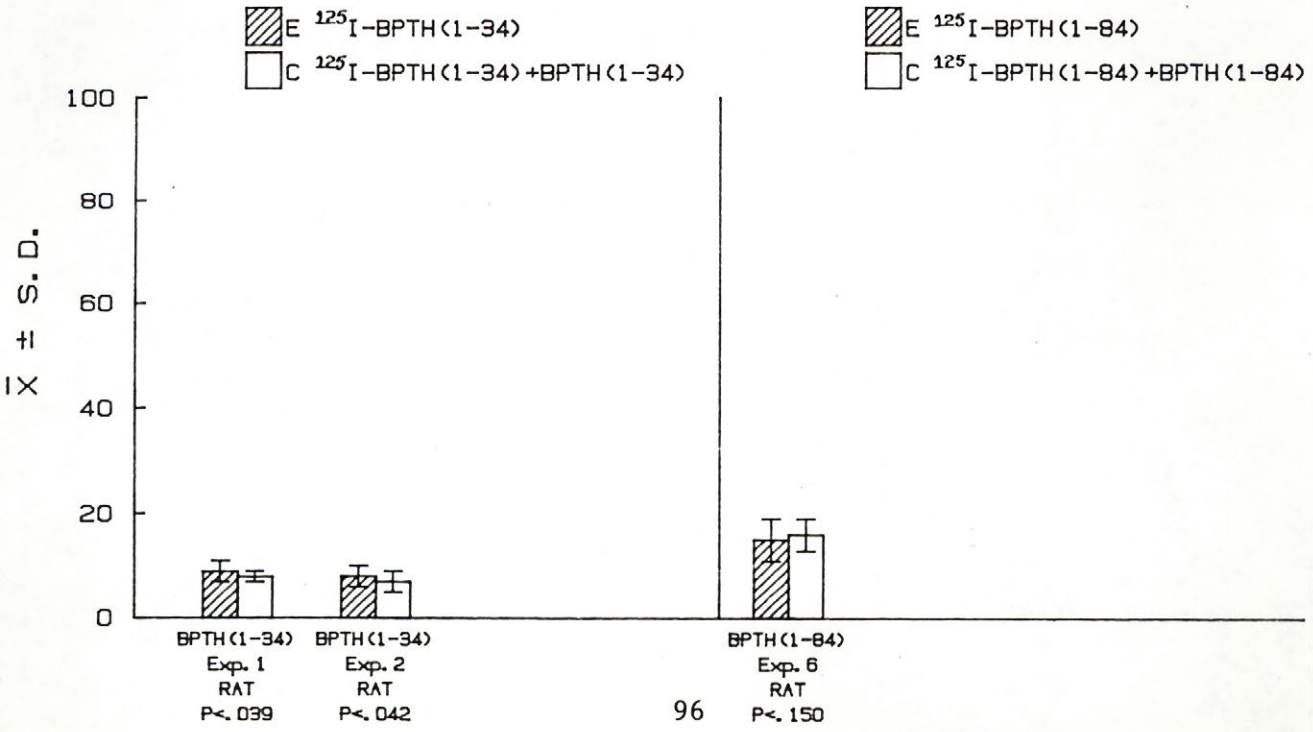
There was a 43 day interval before the coating of the sections with the radioautographic emulsion. X600. ms, mixed spicule.


ms
58
s
59
ms
60
s
61

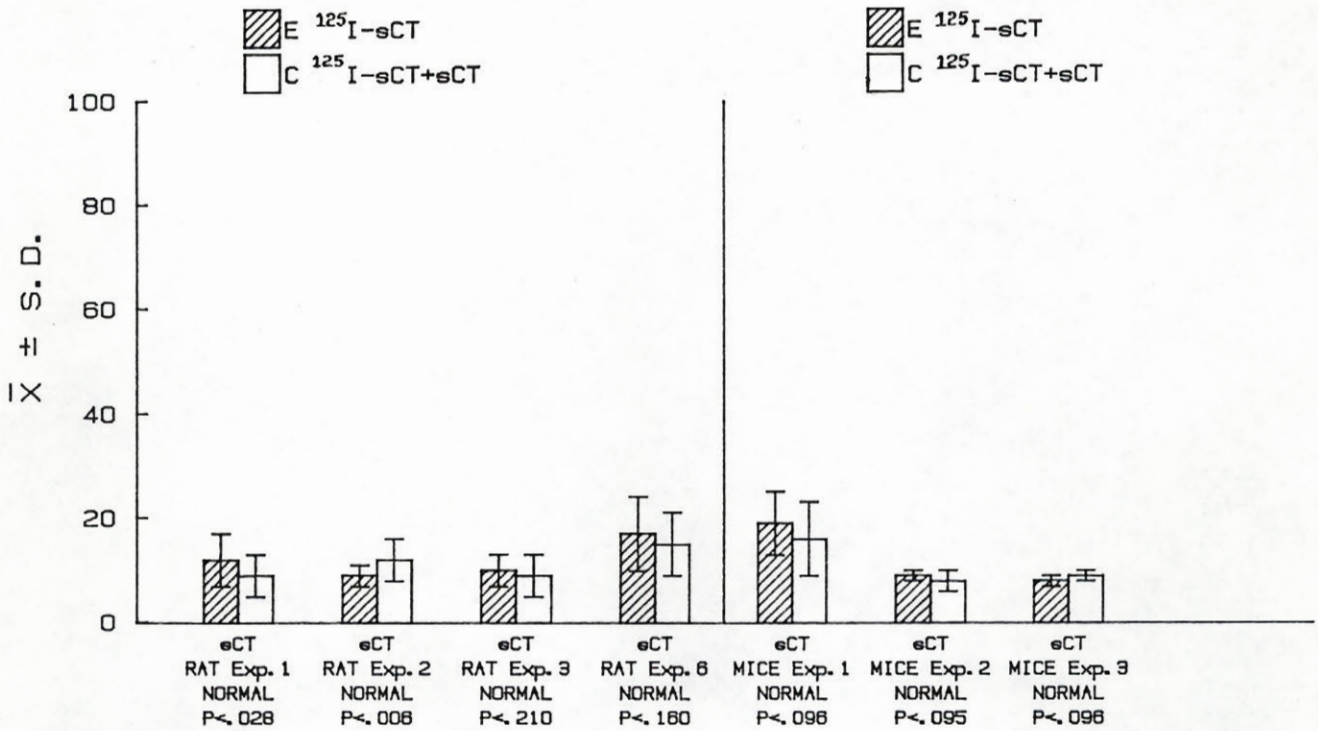
GRAPH 4. **Binding of ^{125}I-bPTH related to <u>osteoblasts</u> in <u>osseous tissue</u> of normal rats.**



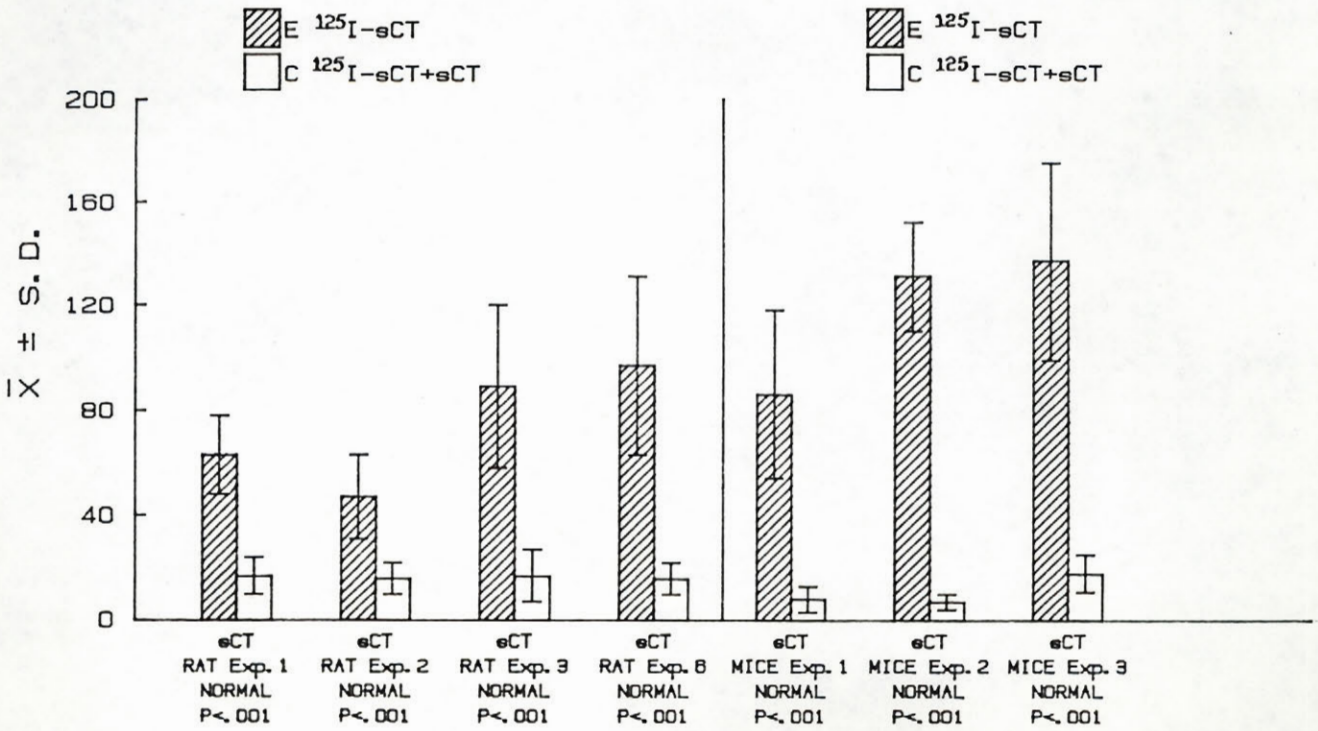
GRAPH 5. **Binding of ^{125}I-bPTH related to <u>osteoclasts</u> in <u>osseous tissue</u> of normal rats.**



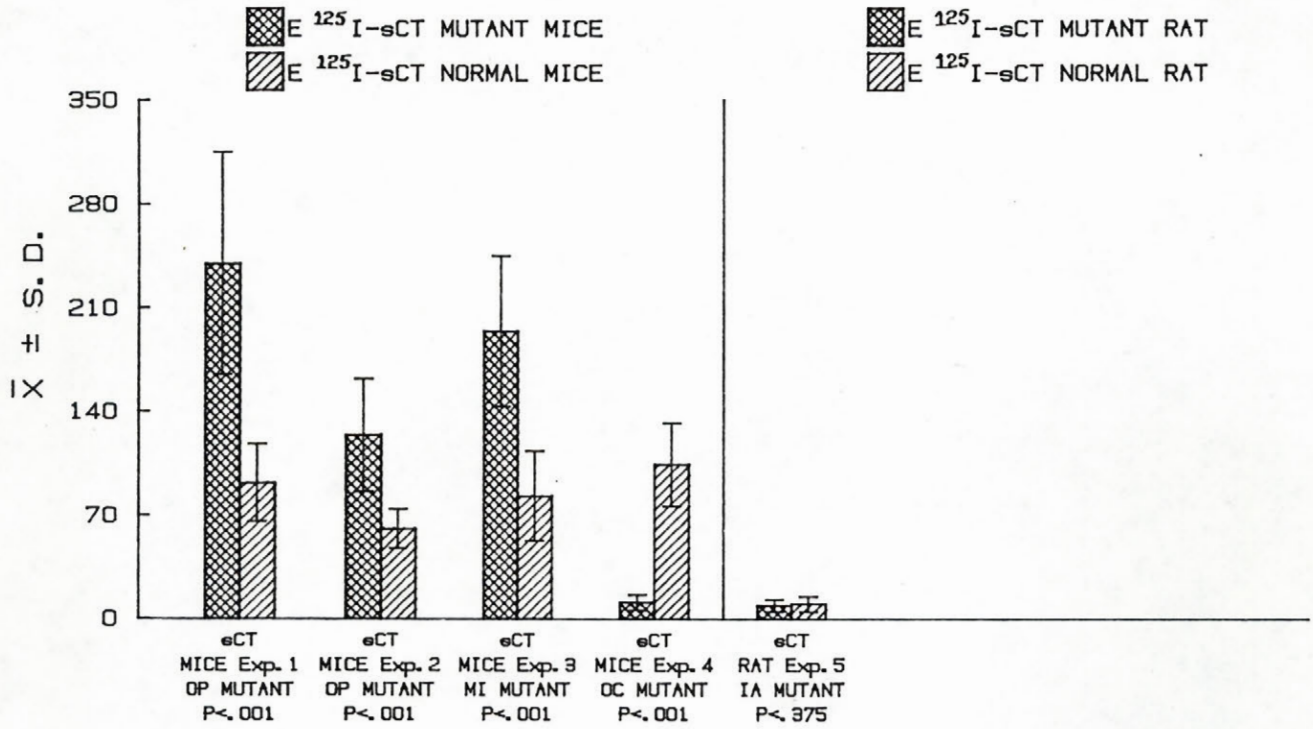
GRAPH 6. Binding of ^{125}I-sCT related to <u>osteoblasts</u> in <u>osseous tissue</u> of normal rodents.



GRAPH 7. Binding of ^{125}I-sCT related to <u>osteoclasts</u> in <u>osseous tissue</u> of normal rodents.



GRAPH 8. **Binding of ^{125}I-sCT related to <u>osteoclasts</u> in <u>osseous</u> <u>tissue</u> of <u>osteopetrotic</u> <u>mutant</u> rodents.**



In Vivo Binding of PTH and CT in the Renal Tissue of Normal Rodents

The kidney is composed of over a million structurally and functionally similar units, the nephrons. Binding for ^{125}I-bPTH and ^{125}I-sCT has been determined in the different portions of the functional unit. The silver grain counts have been reported as over the renal corpuscle (Graphs 9,10) and over the proximal (Graphs 11-16), as well as distal (Graphs 17-28) portions of the tubular nephron. ^{125}I-sCT binding in the renal tissue of the osteopetrotic mutant rodents was estimated (Graphs 29-35) and illustrated (Figs.76, 81-101).

Binding of ^{125}I-bPTH to the renal corpuscle

In light microscope radioautographs made from tissues obtained from Experimental animals injected with ^{125}I-bPTH a strong radioautographic reaction was associated with the renal corpuscles (Fig. 62A). This reaction was absent in the tissues of animals injected with ^{125}I-sCT (Fig. 63A). In corresponding radioautographs obtained from Control animals, ^{125}I-bPTH binding was significantly decreased by co-injection of unlabeled bPTH ($P<0.001$; Graph 9), but not by unlabeled insulin ($P<0.321$; Table 5), whereas, the weaker reaction over glomeruli in animals injected with ^{125}I-sCT was not statistically decreased by co-injection of unlabeled related hormone ($P<0.478$; Graph 10).

In electron microscope radioautographs, the majority of the filamentous grains were found over the plasma membrane of the primary foot processes of the podocytes forming the visceral layer of Bowman's capsule (Figs. 64-66). Some of the silver grains were also distributed over the mesangial cells (Figs 65, 66) and over cells in the kidney interstitium (Fig. 65). In corresponding radioautographs obtained from the

Control animals, the binding of ^{125}I-bPTH to the podocytes foot processes was inhibited maximally by co-injection of excess unlabeled bPTH.

Binding of ^{125}I-bPTH and ^{125}I-sCT to the proximal tubule

A particularity of the binding of iodinated polypeptide hormones to the proximal aspect of the tubular portion of the nephron, was the presence of both luminal and antiluminal reactions. The luminal reaction was strong at short time exposure intervals (1 week) , whereas the antiluminal reaction was detected only at longer time intervals (3 to 6 weeks). In light microscope radioautographs of renal tissue obtained from Experimental animals, the radioautographic reaction was present when ^{125}I-bPTH (Fig. 62B) and ^{125}I-sCT were injected in rats (Fig. 63B), as well as in normal mice (Fig. 75B). The antiluminal reaction over the proximal

tubular portion of the nephron was present when ^{125}I-bPTH was injected (Figs. 62B-D), but absent when ^{125}I-sCT was injected (Figs. 63B-D).

Statistical analysis of the data indicated a significant decrease in the ^{125}I-bPTH antiluminal reaction in radioautographs obtained from the Control animals (P, variable values; Graphs 11-13). The decrease was especially well demonstrated in segment 2, where the strong luminal reaction which obscured the more moderate antiluminal reaction was absent ($P < 0.001$; Graph 12). ^{125}I-bPTH binding was not inhibited by co-injection with unlabeled insulin ($P < 0.202$; Table 5).

In electron microscope radioautographs two minutes after intravenous injection of ^{125}I-bPTH the silver grains were found over the luminal and the antiluminal aspect of the tubular cells in segment 1 (Fig. 68). The antiluminal reaction was associated with the basolateral

membrane invaginations in all three segments of the proximal tubule (Figs. 68,69) and was maximally decreased in radioautographs obtained from animals co-injected with excess unlabeled bPTH. The strong luminal reaction associated with various elements found at the cell apex in segment 1 of the proximal tubular cells following the injection of ^{125}I-sCT in rats (Fig. 71) and in mice (Fig. 77), was identical in density and in distribution to that observed in tissue of animals injected with ^{125}I-bPTH (Fig. 68).

Binding of ^{125}I-bPTH and ^{125}I-sCT to the distal tubule

In light microscope radioautographs obtained from animals injected with ^{125}I-bPTH (Fig. 62E-J) and ^{125}I-sCT in rats (Fig. 63E-J) and in normal mice (Fig. 75E-J), the radioautographic reaction was associated with various cells in the distal tubular portion of the nephron.

Quantitation of the reaction, followed by statistical analysis, indicated that ^{125}I-bPTH binding was significantly decreased in the ascending thick limb ($P<0.05$, in most experiments; Graphs 17-19), in the distal convoluted tubule (Graph 20), in the connecting tubule (Graph 21), as well as in the arched collecting duct (Graph 22). The ^{125}I-bPTH reaction was not significantly decreased by co-injection of unlabeled insulin (Table 5).

Binding of ^{125}I-sCT was significantly decreased in the ascending thick limb ($P<0.001$; Graph 25), in the distal convoluted tubule (Graph 26), in the connecting tubule (Graph 27), and in the arched collecting duct (Graph 28), by a simultaneous injection of unlabeled sCT. Binding of ^{125}I-sCT was not prevented by excess unlabeled bPTH(1-34) (Table 6), nor by excess unlabeled insulin (Table 8).

At the electron microscope level, the reaction due to ^{125}I-bPTH binding was associated with the antiluminal aspect of the DCT-cell of the ascending thick limb (not illustrated). Occasionally, a low reaction was observed related to a cell of the late portion of the distal nephron which resembled in description the dark or intercalated cell (Fig.70).

Binding of ^{125}I-sCT was intense over the antiluminal aspect of DCT-cells in rats (Fig. 72) and in mice (fig. 78). Binding was equally related to the CNT-cell (Fig. 73), and to the dark or intercalated cells (Fig. 79). Additionally, as for ^{125}I-bPTH, a low amount of reaction was related to cells in the wall of the latter portion of the distal tubular portion of the nephron, presumably, intercallated cells based on morphological criteria (Fig. 74, in rats; Fig. 80, in mice).

In Vivo binding of CT in Renal Tissue of Osteopetrotic Rodents

Osteopetrotic and Microphthalmic mice

Qualitative and quantitative analysis of the radioautographic reaction obtained in kidney tissue from osteopetrotic mutant rodents injected with ^{125}I-sCT suggested major alterations in ^{125}I-sCT binding, as well as in cellular ultrastructure.

Binding of ^{125}I-sCT to the proximal tubule

In normal mice two minutes after intravenous injection of ^{125}I-sCT a strong non-competitive reaction (Fig 75B) was observed associated with ultrastructural elements located at the apex of the cells forming the walls of the early proximal tubular segment (Fig. 77). In light microscope radioautographs obtained from osteopetrotic op/op mice this

reaction was absent (Fig. 76B).

Quantitation and statistical analysis indicated a significant decrease in the luminal reaction in the op/op mutants when compare with the reaction in the phenotypically normal siblings ($P<0.001$; Graph 29). A small amount of reaction was observed at the neck region of the proximal tubule (Fig. 76A). The decrease in reaction was paralleled by ultrastructural alterations in the proximal tubular cells (Figs. 81-83). Among the abnormalities was an increase in the number of dense cytoplasmic bodies distributed at the apex of the cells just below the brush border at the region where normally one can find a large number of all sizes of vesicles and vacuoles.

Whereas in osteopetrotic op/op mice the strong proximal tubular luminal reaction was virtually absent (Fig. 76B), in the microphthalmic mice it appeared overly intense (Fig. 85B) and statistical analysis indicated a significant increase in the reaction in the microphthalmic mice when compared to the reaction obtained in the normal littermate ($P<0.001$; Graph 29).

In electron microscope radioautographs, although some of the cross sections in segment 1 of the proximal tubule had normal morphology (Fig. 86), a great portion were altered (Figs. 87,89). In mi/mi mutants, the intense reaction in segment 1 was related to the cells showing decreased height, large irregularly shaped mitochondria with peculiar nucleolar structures (Fig. 87). Additionally, in segment 3 there was a high number of dense cytoplasmic bodies (Fig. 89).

Binding of ^{125}I-sCT to the distal tubule

In normal mice ^{125}I-sCT bound to the antiluminal aspect of

cells in the distal tubule (Fig. 75E-J). In osteopetrotic op/op and in microphthalmic mi/mi mice (Fig. 85E-j), the distribution of the binding appeared identical to that observed in their phenotypically normal siblings.

Quantitation and statistical analysis of the reaction obtained in these two mutants indicated no significant difference between the reaction observed in the mutants and that obtained in the phenotypically normal siblings (Graphs 30-32).

Osteosclerotic and incisor absent mutant rodents

Binding of ^{125}I-sCT to the proximal tubule

In osteosclerotic oc/oc mice, the radioautographic reaction appeared normal, but slightly lower in incisor absent ia/ia rats (Graphs 29; experiments 4 and 5).

Electron microscope observations of ultrastructure in the proximal tubular portion of the nephrons in oc/oc mice, although not quantitative indicated that vacuoles at the apex of the cells of segments 1 were very small, or abnormally large (Figs. 92, 93). Also there was an increase in the number of dense cytoplasmic bodies in segment 3 of this tubular portion of the nephron (Fig. 94). Similarly, in the incisor absent ia/ia rats the cells in the wall of the earliest segment of the proximal tubule contained an abnormally large amount of dense cytoplasmic bodies with a reduction in the apical canalicular system (Fig. 98). In the latter segment some of the cells were disrupted and contained small numbers of mitochondria. The number of larger vacuoles was greatly decreased in the early segment and abnormally increased in the latter segment.

Binding of ^{125}I-sCT to the distal tubule

In light microscope radioautographs the ^{125}I-sCT distal tubular reaction was greatly decreased. This decrease was accompanied by structural abnormalities apparent at the light microscope level (Figs. 90,91).

Statistical analysis indicated a significant decrease ($P<0.002$; Graphs 30-32) in the distal tubular reaction in the oc/oc and in the ia/ia osteopetrotic mutant rodents.

At the electron microscopic level, the cells found in the walls of the distal tubule in the oc/oc mice (Figs. 95-97) showed an increase in basal invaginations together with smaller, longer mitochondria closely related to the basal invaginations; an arrangement seldom observed in phenotypically normal littermates (Figs. 95-96). In intercalated cells, there was a large number of small uncoated vesicles localized in the bulging apex of these cells. In the incisor absent ia/ia rats, the cells found in the walls of the distal tubule were irregular. They contained a large number of ribosomes, whereas the number of mitochondria and uncoated vesicles was greatly decreased (Figs. 100,101).

There was no major difference in ^{125}I-sCT binding when male was compared with female except for a slight increase in binding in the liver of the female animal (Table 9).

PLATE 16

Binding of parathyroid hormone and calcitonin in the nephron of normal rats. Light microscope radioautography.

Figure 62 A-J Binding of ^{125}I-bPTH(1-84) in Experimental animal. BPTH(1-84) Exp 1. Exposure 3 weeks (A,C-J) and 1week (B).

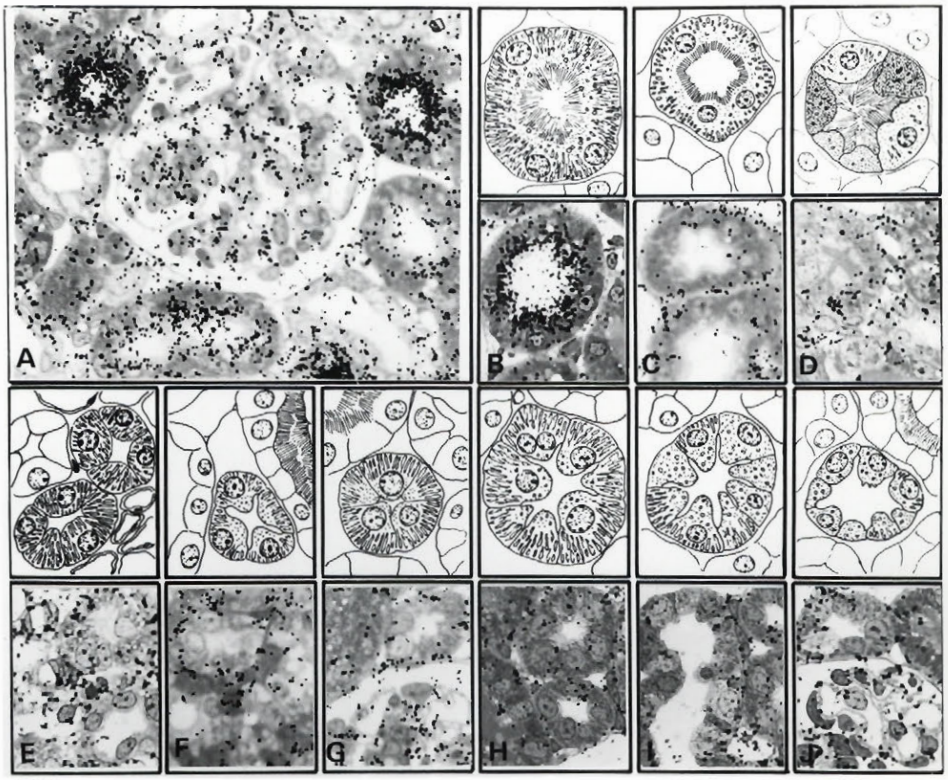
Figure 63 A-J Binding of ^{125}I-sCT in Experimental animal. SCT Exp 7. Exposure 2 weeks (A,C-J) and 1 week (B).

In the light microscope radioautographs obtained from kidneys, competitive specific binding for ^{125}I-bPTH (Fig. 62) is related to the renal corpuscle (A), to the antiluminal aspect of the three segments of the proximal tubular portion of the nephron (B-D), and to the thick ascending limb as it courses through the outer medulla (E,F) and the cortex (G). Beyond the macula densa the reaction is low (H-J), especially in the connecting tubule (I) as illustrated in the montage. Non-competitive binding of high density appears on the luminal aspect of segment 1 of the proximal tubule (B).

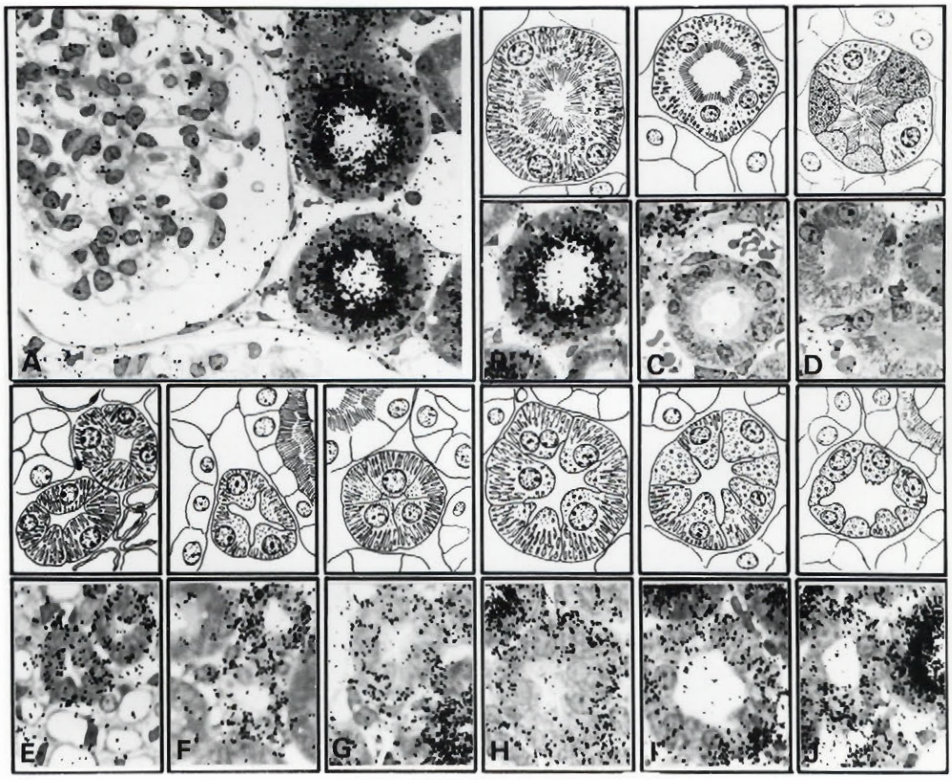
In animals injected with ^{125}I-sCT (Fig. 63), competitive specific binding is found related to the antiluminal surface of all segments of the distal tubular portion of the nephron. These include the thick ascending limb as it passes through the outer medulla (E,F) and the cortex (G), the distal convoluted tubule (H), the connecting tubule (I), and the arched collecting tubule (J). Similar to the binding of ^{125}I-bPTH, non-competitive binding for ^{125}I-sCT appears over the luminal aspect of segment 1 of the proximal tubule (B).

In light microscope radioautographs obtained from the Control animals, the renal corpuscle reaction and all antiluminal binding to the tubular portion of the nephron is blocked by co-injection of excess related unlabeled hormone.

There was a 35 day interval before coating of the sections with the radioautographic emulsion. X450.



62



63

PLATE 17

Binding of parathyroid hormone
in the renal corpuscle and in kidney interstitium
in normal rats.
Electron microscope radioautography.

Figures 64-67 Binding of bPTH(1-84) in Experimental animals.
BPTH(1-84) Exp 6. Exposure 120 days.

In animals injected with ^{125}I-bPTH(1-34) or ^{125}I-bPTH(1-84), the filamentous silver grains are found over the primary foot processes (fp) of the podocytes (P) forming the visceral layer of Bowman's capsule (Figs. 64-66). A small amount of grains is related to the Golgi of these cells (Fig. 64; single arrow). When ^{125}I-bPTH(1-84) was injected, silver grains were observed over the mesangial cells (M) (Figs. 65, 66). The binding of ^{125}I-bPTH to the primary foot processes is inhibited by co-injection of excess unlabeled homologous hormone.

Additionally, ^{125}I-bPTH(1-84) binds to kidney cells found in the cortical interstitium (Fig. 67). The cells resemble type 2 cortical interstitial cells, as they contain numerous lysosomes (fig 67; single arrow) and phagocytic vacuoles, as well as the usual organelles.

There was a 68 day interval before coating of the sections with the radioautographic emulsion. X8,250. bv, blood vessel.

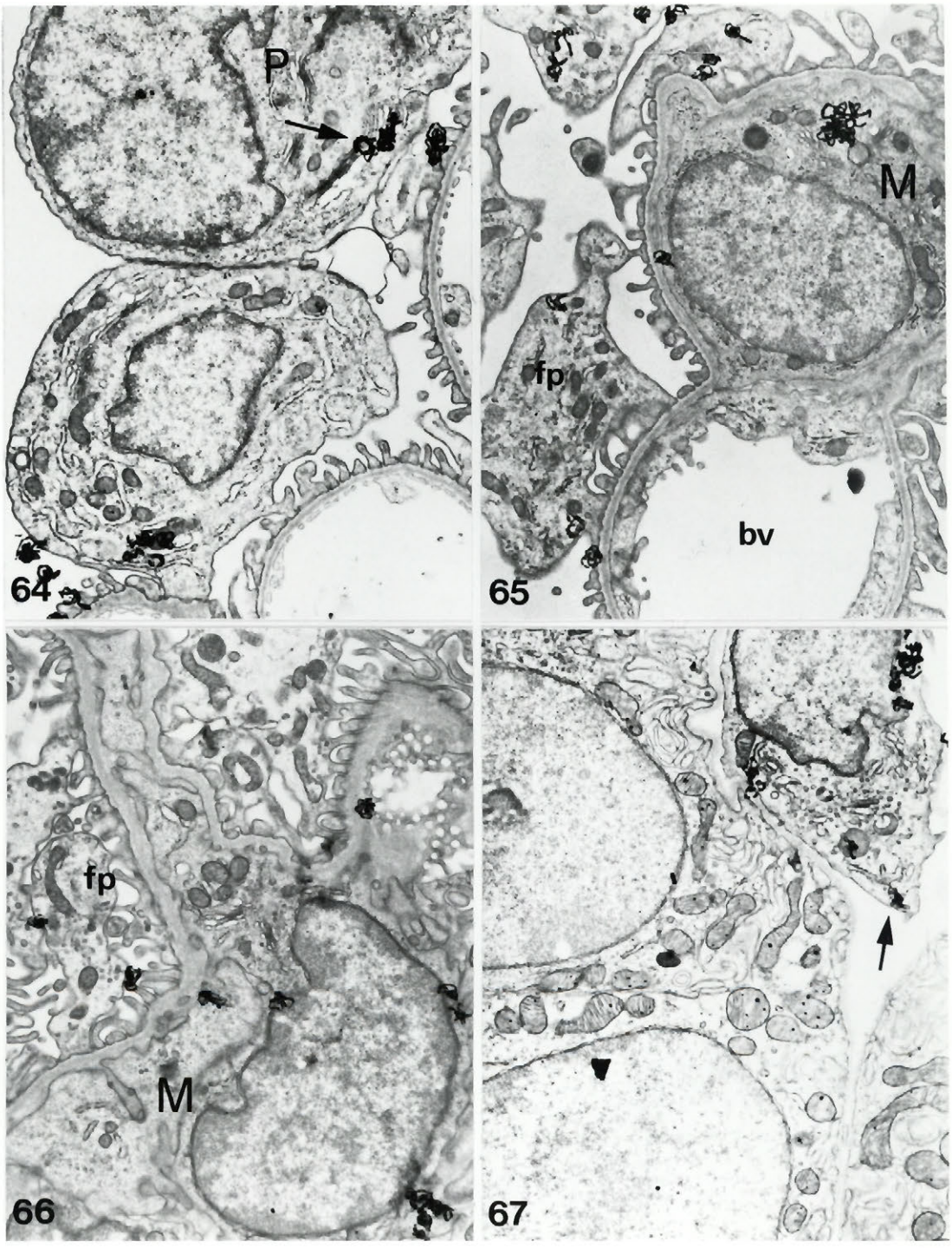

P
64
M
fp
bv
65
fp
M
66
67

PLATE 18

Binding of parathyroid hormone to the tubular portion of the nephron in normal rats.
Electron microscope radioautography.

Figures 68-70 Binding of ^{125}I-bPTH(1-84) in Experimental animals. BPTH(1-84) Exp 6. Exposure 120 days.

Two minutes after the intravenous injection of ^{125}I-bPTH(1-84), silver grains are found on both luminal and antiluminal aspects of cells forming the walls of segment 1 of the proximal tubule (Fig. 68). The luminal reaction is associated with the brush border (BB), the coated pits and vesicles (single arrow), and the membrane of empty vacuoles of variable sizes (double arrow), as well as that of cytoplasmic dense bodies (triple arrows). The antiluminal reaction is associated with the basolateral cell membrane invaginations of all the segments of the proximal tubule. The binding is higher in segment 1 (Fig. 68) and in segment 2 (Fig. 69) where a substantial amount of basal membrane invaginations correlates with increase in cell surface.

In the distal portion of the nephron, an antiluminal reaction is associated with the DCT-cells which form the walls of the thick ascending limb and that of the distal convoluted tubule (not illustrated). Some silver grains are related to the cells which probably develop from the uteric bud (Fig. 70). These resemble the intercalated cells but do not fit exactly its classical description.

The antiluminal reaction is completely inhibited by co-injection of excess homologous unlabeled hormone. The luminal reaction appears unchanged.

There was a 68 day interval before coating of the sections with the radioautographic emulsion. X8,250. cb, cytoplasmic dense body.

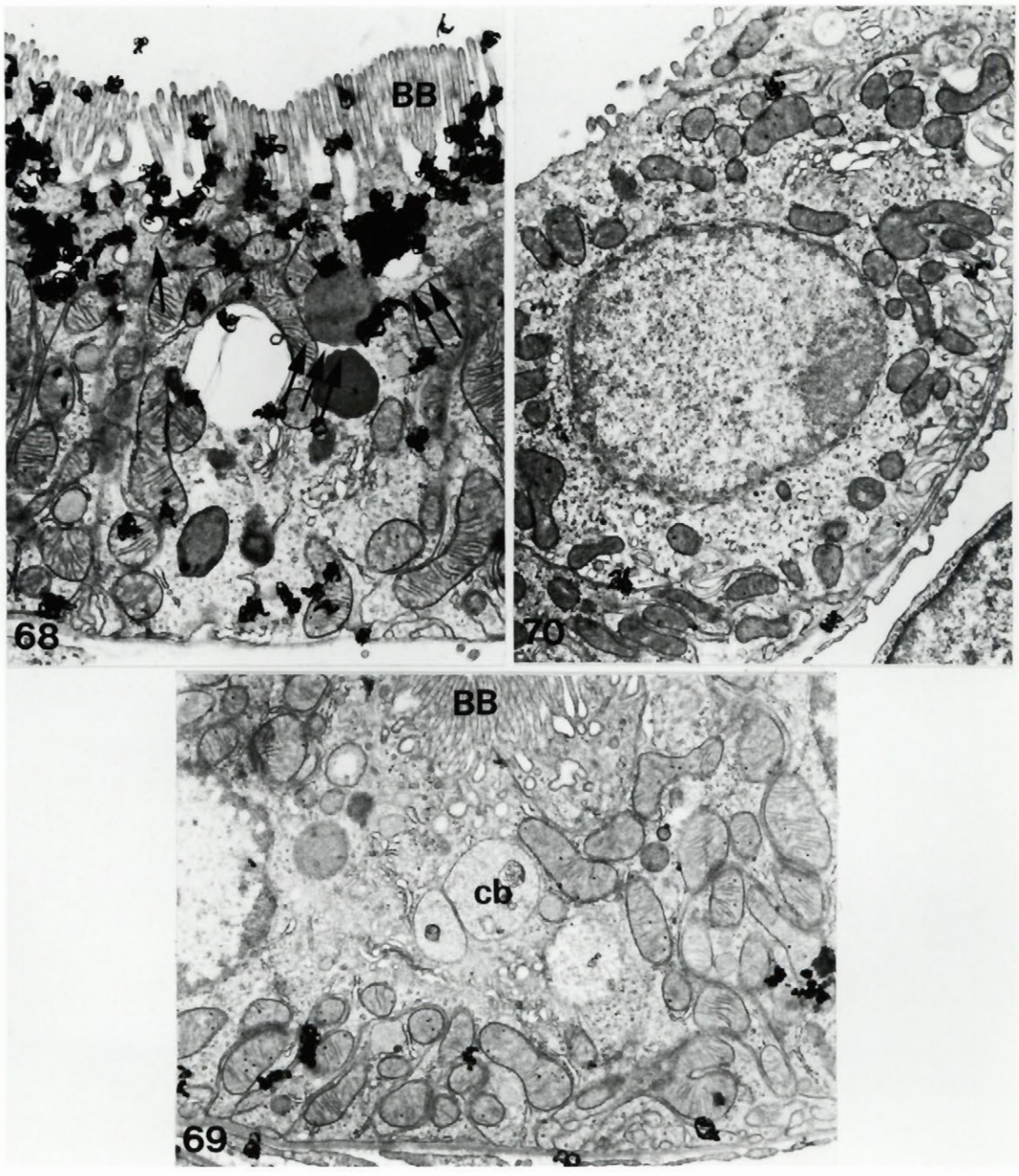

BB
68
70
BB
cb
69

PLATE 19

Binding of calcitonin to the tubular portion of the nephron of normal rats.
Electron microscope radioautography.

Figures 71-74 Binding of ^{125}I-sCT in Experimental animals.
SCT Exp 7 Normal rats. Exposure 80 days.

Competitive specific binding for ^{125}I-sCT is found over the distal tubular portion of the nephron. The non-competitive radioautographic reaction at the apex of the proximal tubular cell is identical in distribution to that observed for ^{125}I-bPTH (Fig. 68). The silver grains are related to the brush border (Fig. 71), to coated pits and vesicles (single arrow), to the membrane of variable sized vacuoles (double arrow)s, and to cytoplasmic dense bodies (triple arrow s)

In normal rats, the antiluminal reaction is found on the basal aspect of DCT-cells forming the walls of the thick ascending limb and a portion of the walls of the distal tubule beyond the macula densa (Fig. 72). The cells are recognized by the absence of brush border and the presence of numerous basal membrane invaginations, housing long perpendicularly arranged mitochondria.

Substantial binding is also associated with the CNT-cells found in the wall of the connecting tubule (Fig. 73). This cell is recognized by its transparency and the reduced amount of basal membrane infoldings, as well as the presence of a small amount of round to oval mitochondria.

A low number a silver grains is associated with what could be the intercalated cells (Fig. 74). The cell is recognized by the rigid cell surface projections (Fig. 74; single arrow), and the presence of mitochondria distributed randomly in an oval to round shaped cell.

There was a 69 day interval before the coating of the sections with the radioautographic emulsion. X7,125.

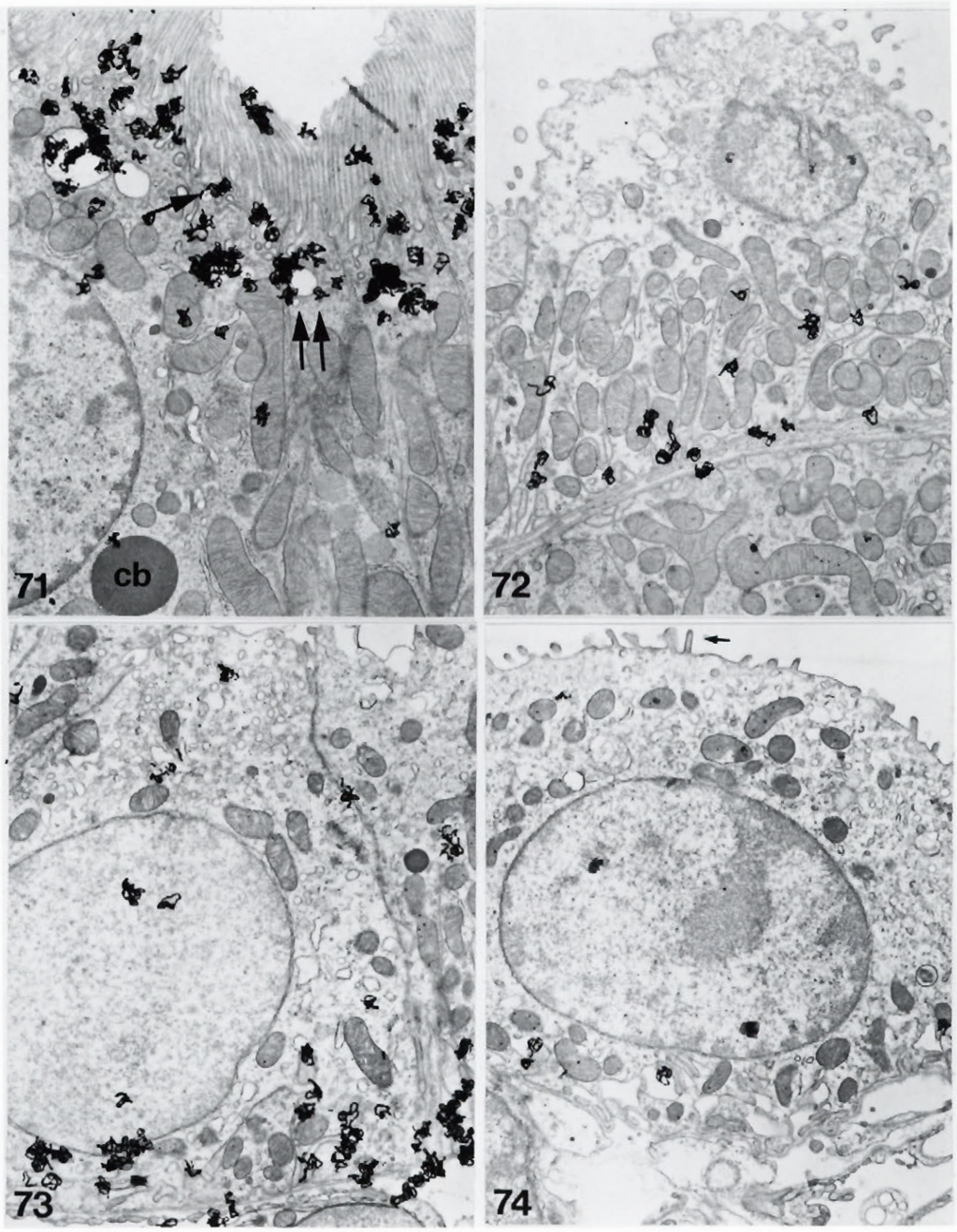

71
cb
72
73
74

PLATE 20

Binding of calcitonin in the nephron
of normal and osteopetrotic op/op mice.
Light microscope radioautography

Figure 75 A-J — Binding of sCT in Experimental animal. SCT Exp 2 Normal mice. Exposure 1 week (B), and 2 weeks (A, C-J).

Figure 76 A-J — Binding of sCT in Experimental animal. SCT Exp 2 OP Mutant mice. Exposure 1 week (B), and 3 months (A, C-J).

In radioautographs obtained from normal mice (Fig. 75) injected with ^{125}I-sCT, competitive specific binding is associated with the antiluminal aspect of the thick ascending limb as it courses through the outer medulla (E,F) and the cortex (G), as well as to the distal convoluted tubule (H-J).

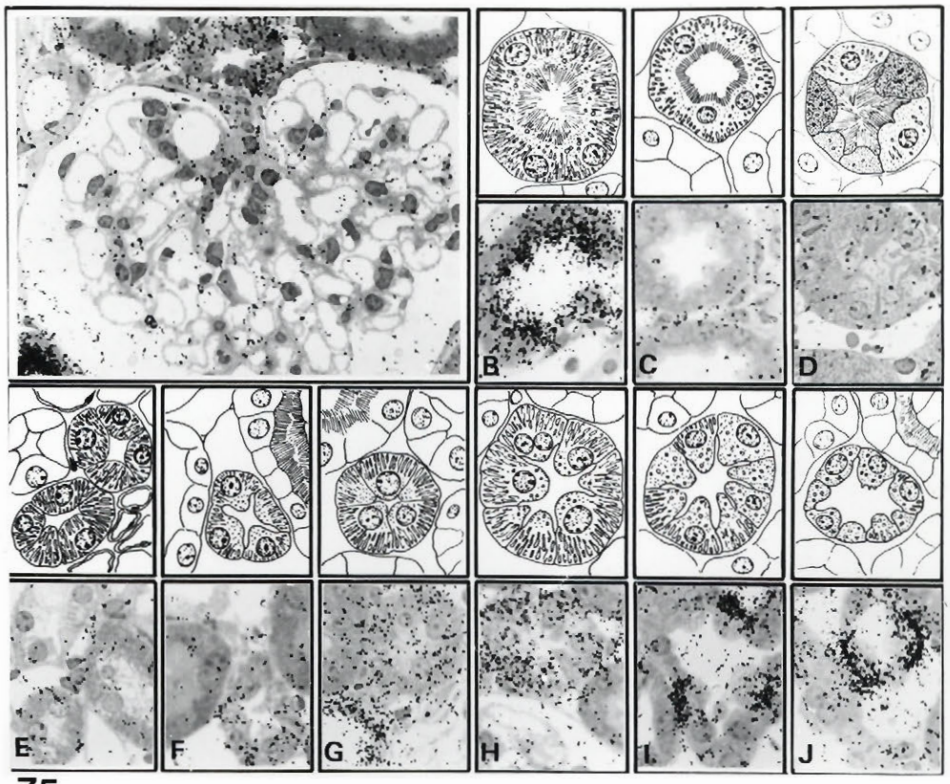
The reaction is strong over cells which under light microscopic observations appear clear, and over cells that appear dark in the latest segment of the distal nephron (I, J).

As usual after injection of labeled polypeptide hormones, a strong non-competitive reaction is present in segment 1 of the proximal tubular portion of the nephron (B).

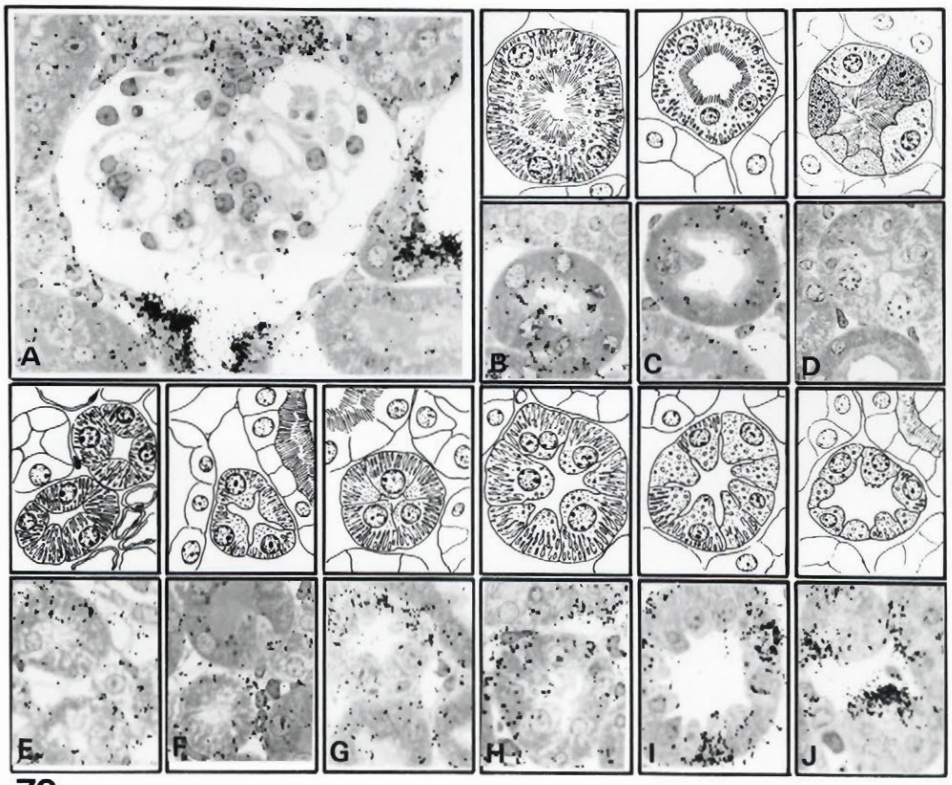
There is no difference in the distribution of the competitive specific binding in renal tissue obtained from op/op mice (fig. 76) when compare to that of normal mice (Fig 75). It is observed on the antiluminal aspect of most cells of the distal portion of the nephron (E,J).

The typical strong non-competitive luminal reaction observed in segment 1 of of the proximal tubule in normal rats (Fig. 63B), and normal mice (Fig. 75B), is greatly decreased in the osteopetrotic op/op mice (Fig. 76B). When present, it may be seen related to the neck of the proximal tubule (Fig. 76A; arrow).

There was a 36 day (Fig. 75), and an 18 day (Fig. 76) interval before coating of the sections with the radioautographic emulsion. X450.



75



76

PLATE 21

Binding of salmon calcitonin to the tubular portion of the nephron of normal mice.
Electron microscope radioautography

Figures 77-80 Binding of ^{125}I-sCT in Experimental animals.
SCT Exp 3 Normal mice. Exposure 55 days.

In normal mice, the distribution of ^{125}I-sCT is comparable to that reported in the normal rat (Plate 19). In the apex of the cell forming the walls of segment 1 of the proximal tubular portion of the nephron there is a reduction in the number of empty vacuoles as compared to the rat (Fig. 77). At the ultrastructural level the presence of unusual inclusions have been observed. These include lipid droplets crowned by crescent-shaped electron dense material (Figs. 77; arrow).

The non-competitive luminal reaction is localized exclusively to the apical region of the cell forming the wall of segment 1 of the proximal tubule as described earlier.

The antiluminal reaction is related to DCT-cells forming the walls of the thick ascending limb and those of the distal convoluted tubule (Fig. 78). The reaction is strong over the basal aspect of cells in the connecting tubule (Fig. 79), as well as, the cortical collecting duct (Fig. 80).

Whereas the luminal reaction is not altered by co-injection of excess unlabeled calcitonin, the antiluminal reaction is maximally inhibited in tissue of the Control animals.

There was a 69 day interval before coating of the sections with the radioautographic reaction. X7,125. L, lumen.

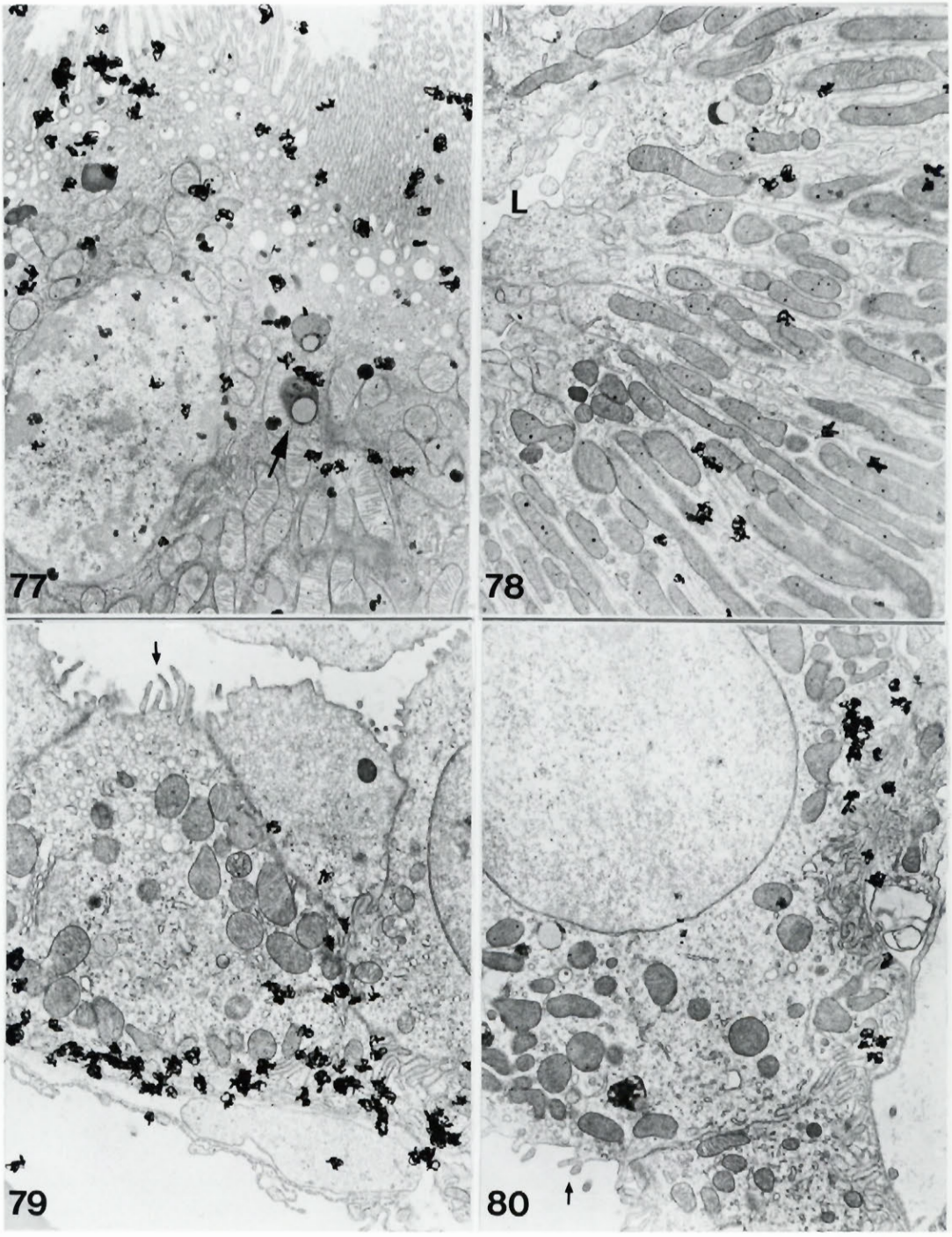

77
L
78
79
80

PLATE 22

Proximal tubule structure in the nephron
of osteopetrotic op/op mice.
Electron microscope radioautography.

Figures 81-83 Binding of ^{125}I-sCT in Experimental animal.
SCT Exp 2 OP Mutant mice. Exposure 80 days.

In osteopetrotic op/op mice, the competitive specific binding is identical in distribution (Plate 21), and intensity (Graphs 30-32) to that seen in phenotypically normal siblings.

The strong luminal reaction observed in segment 1 of the proximal tubule of normal mice (Fig. 77) is absent in electron microscope radioautographs obtained from the osteopetrotic mutants (Figs 81, 82).

The system of canaliculi appears normal, but the vacuolar system is greatly decreased and there is an abnormal amount of dense bodies at the apex of the proximal tubular cells (Figs. 81; single arrow). The mitochondria are often enlarged and abnormally positioned (Figs 82,83; double arrows).

There was a 47 day interval before coating of the sections with the radioautographic emulsion. X7,125.

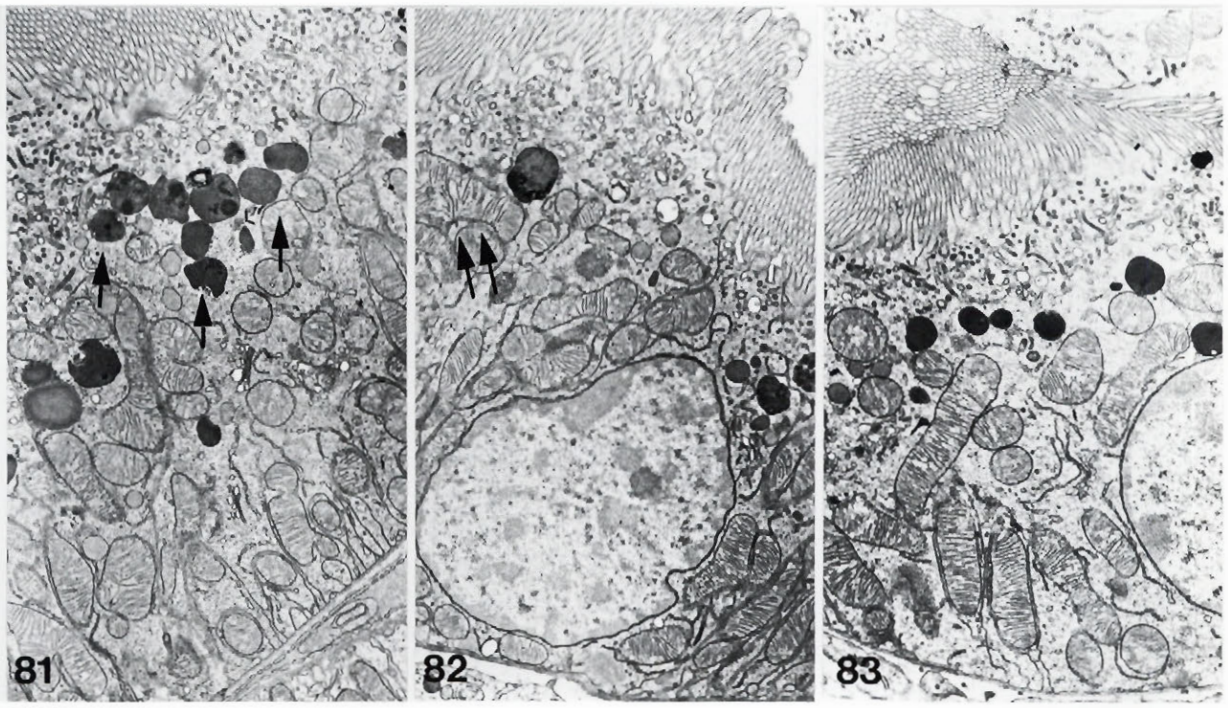

81
82
83

PLATE 23

Binding of salmon calcitonin to the nephron of microphthalmic mi/mi mice. Light microscope radioautography

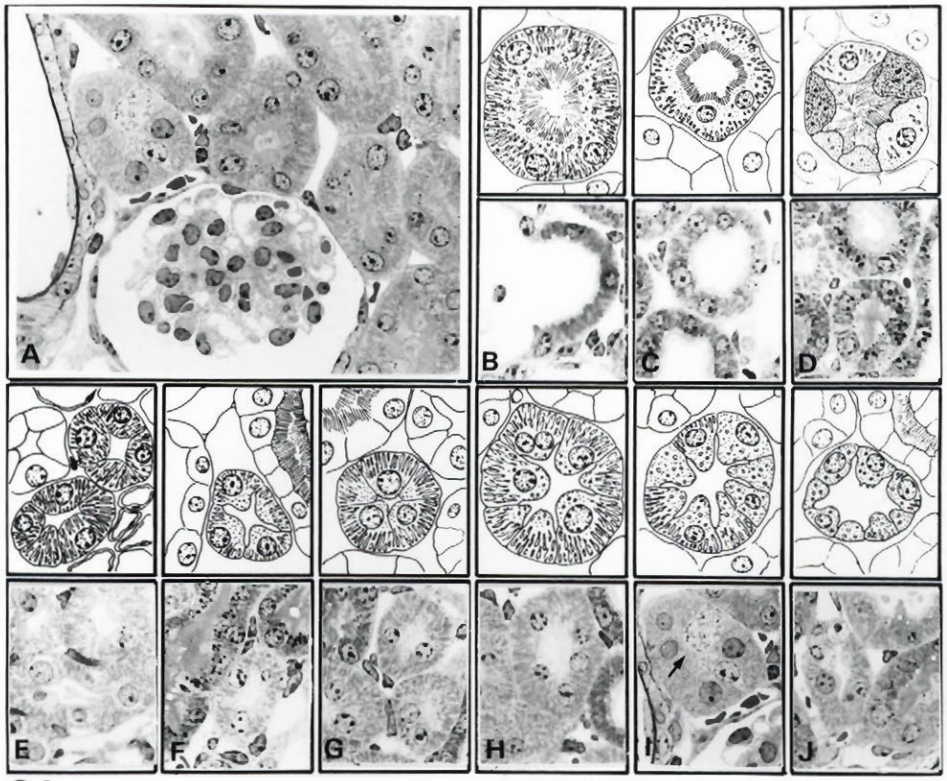
Figure 84 A-J — Light microscope histology of the different segments of the microphthalmic mi/mi mouse nephron. SCT Exp 3 MI Mutant mice. Toluidine blue.

Figure 85 A-J — Binding of ^{125}I-sCT in Experimental animal. SCT Exp 3 MI Mutant mice. Exposure 1 week (B), and 2 weeks (A, C-J).

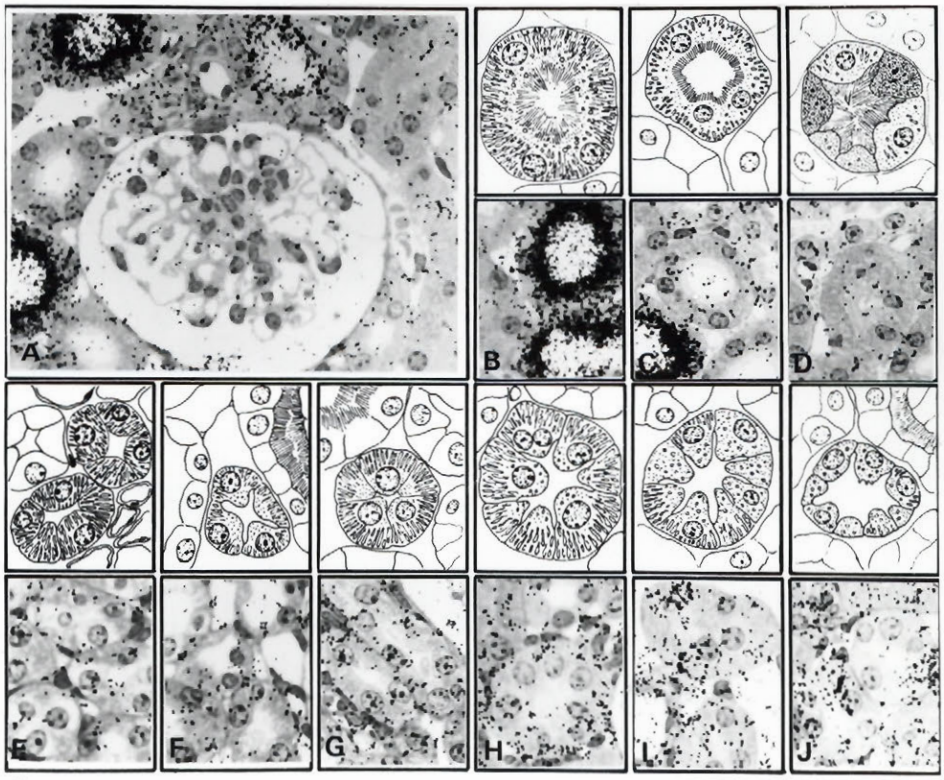
In histological preparation of the kidney obtained from the mi/mi mutant (Fig. 84 A-J), the renal corpuscles look small (A). The cells forming the walls of segment 1 of the proximal tubular portion of the nephrons are abnormally low (B). However, most segments of the distal tubule have a regular histology, with the exception of the CNT-cells which are enlarged (I; arrow).

In the light microscope radioautographs obtained from mi/mi mice injected with ^{125}I-sCT (Fig. 85), the antiluminal competitive specific binding has the same distribution and intensity as in phenotypically normal siblings. An unusually intense radioautographic reaction is observed associated with segment 1 of the proximal portion of the nephron (B).

There was a 47 day interval before the coating of the sections with the radioautographic emulsion. X450.



84



85

PLATE 24

Binding of salmon calcitonin in the proximal tubule portion of the nephron in microphthalmic mi/mi mice. Electron microscope radioautographs*

Figures 86-89 Binding of ^{125}I-sCT in Experimental animal. SCT Exp 3 MI Mutant mice. Exposure 80 days.

Quantitative analysis of the ^{125}I-sCT antiluminal reaction in the mi/mi mutant and its normal sibling indicated no significant difference in reactions (Graphs 30-32). The non-competitive reaction, at the luminal aspect of segment 1 of the proximal tubular portion of the nephron, is significantly increased in the mi/mi mutant (Graph 29; Fig. 85B).

Although a certain proportion of cross sections of the proximal tubule appear normal, with healthy vacuolar structures (Fig. 86; arrows), a great proportion of the cross sections are abnormal (Fig. 87). The alterations in ultrastructure range from abnormally low cell height to decreased and disturbed basal membrane infoldings. The mitochondria are large (single arrow) and abnormally positioned. The nuclei show unusual nucleoli (double arrows).

Segment 2 appears normal (Fig. 88). However, and in segment 3, there is an enormous amount of dense bodies and the basal lamina is greatly increased (Fig. 89).

There was a 47 day interval before the coating of the sections with radioautographic emulsion. X7,125.

* Fig. 87, only.

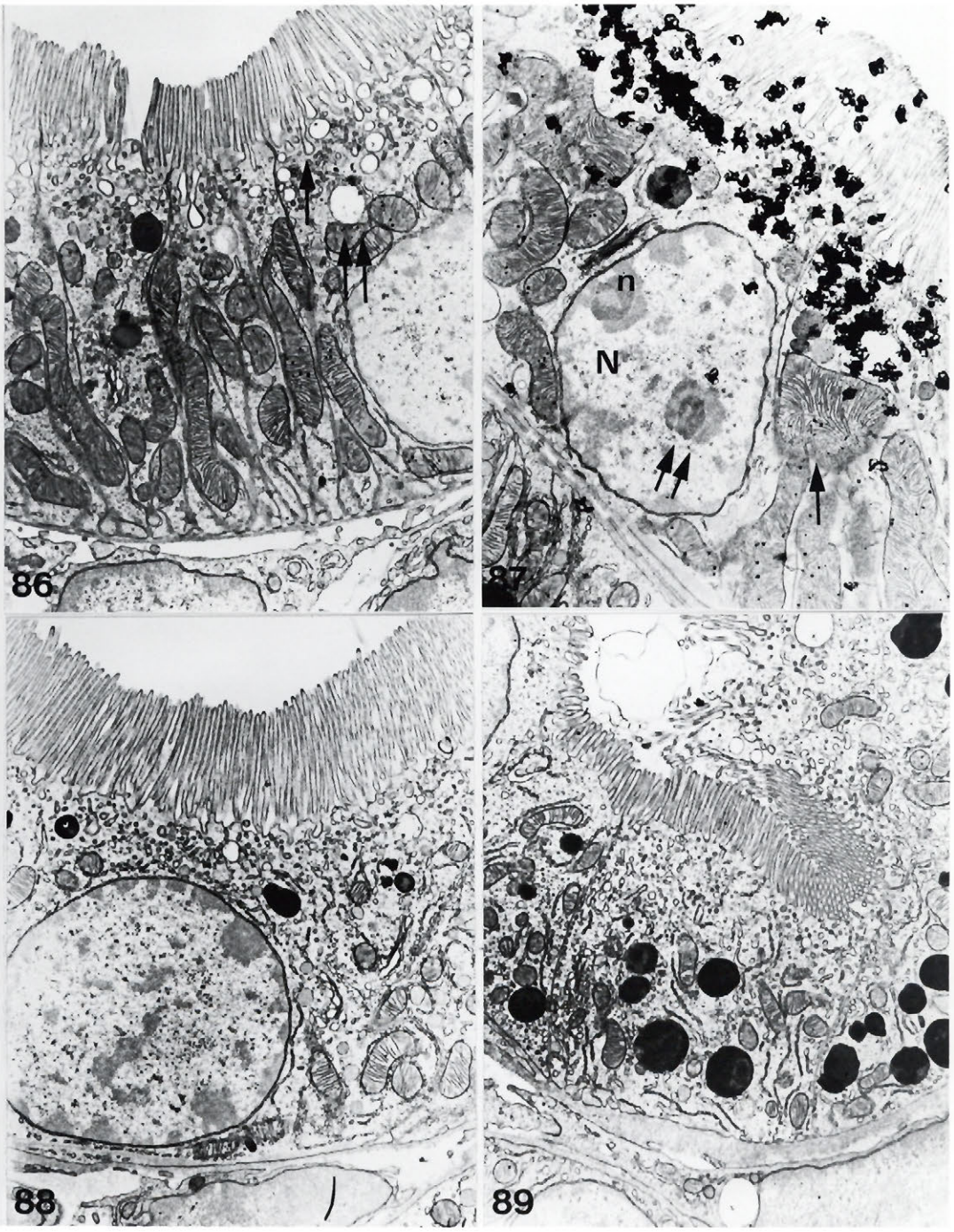

86
n
N
87
88
89

PLATE 25

Light microscope

Segmentation and histology of the nephron

in osteosclerotic oc/oc and incisor absent ia/ia rodents.

Figure 90 A-J Light microscope histological preparation of renal tissue obtained from osteosclerotic oc/oc mutant mouse. SCT Exp 4 OC Mutant mice.

Figure 91 A-J Light microscope histological preparation of renal tissue obtained from incisor absent ia/ia rats. SCT Exp 5 IA Mutant rat.

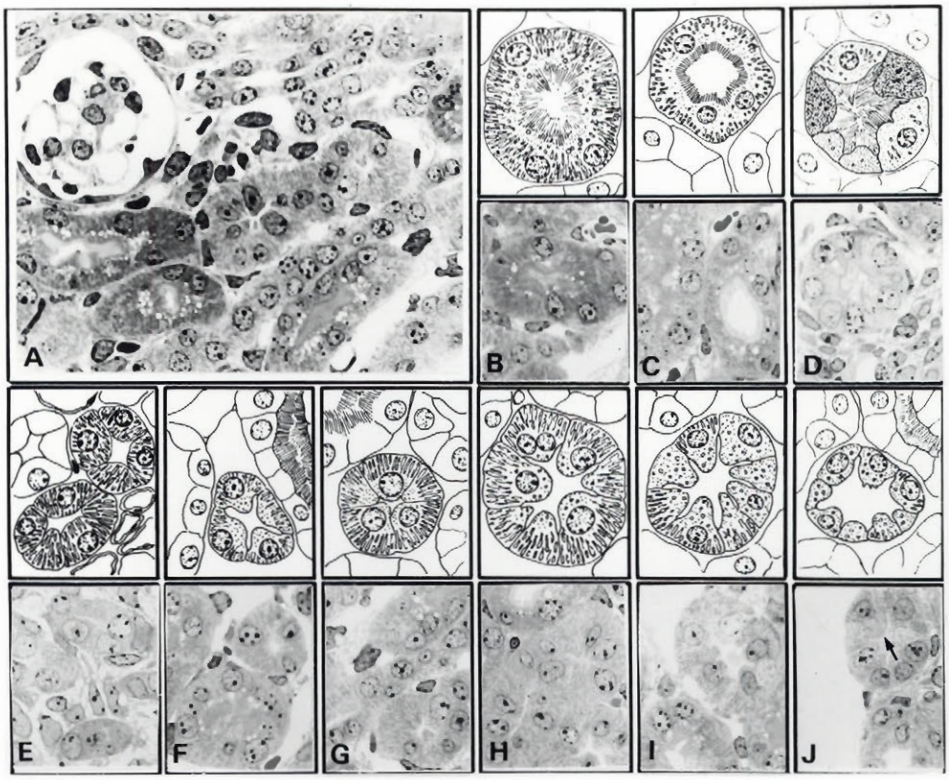
Many structural abnormalities are observed in histological preparations of kidney tissues obtained from osteosclerotic oc/oc mice (Fig. 90A-J) as well as from the incisor absent rats (Fig. 91A-J).

The renal corpuscles are small and the podocytes occasionally have rather dark nuclei, scarce cytoplasm, and no evident cytoplasmic processes.

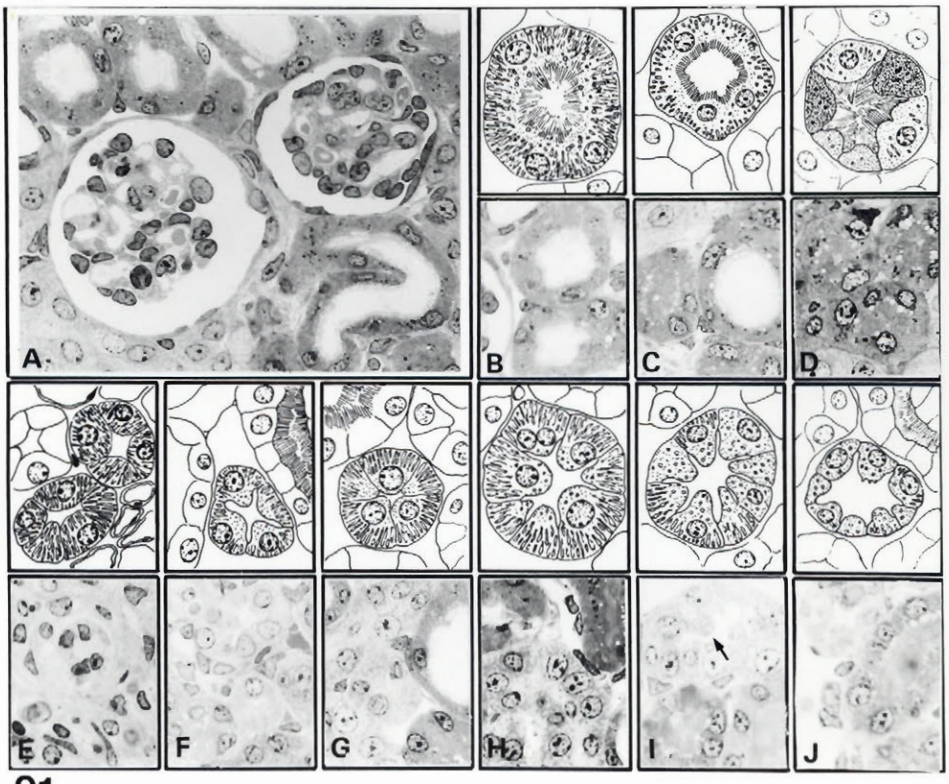
The proximal tubular cells show a decrease in basal striations, particularly in the incisor absent mutant (Figs. 91B-D). The luminal endocytotic system, normally composed of variable sized vacuoles and localized usually to segment 1, is also found in segment 2. In this location, it is composed of abnormally large vacuoles (Figs. 90B-D). The brush border in the incisor absent mutant is particularly disrupted and the cell height greatly decreased (Fig. 91B).

In the distal portion of the nephron, the basal striations, which results from numerous infoldings of the plasma membrane especially in the DCT-cells, is not apparent (E-J). The apical portion of some of the cells in the connecting tubule and in the arched collecting duct, staindensely (Figs 90, 91; I-J; arrow).

Generally speaking the degree of disturbance is higher in the incisor absent rat and most remarkable in the ascending thick limb (Fig. 91 E-G). X450.



90



91

PLATE 26

Electron micrographs

Histology of the proximal and distal tubule portions of the nephron of osteosclerotic oc/oc mice.

Figures 92-97 Electron micrographs of renal tissue prepared from osteosclerotic oc/oc mouse.
SCT Exp 4 OC Mutant mice.

In the osteosclerotic oc/oc mouse, the proximal tubular cells in segment 1 are often low and contain a large amount of regularly spaced large vacuoles (Fig. 92, 93; single arrow). The basal membrane infoldings and mitochondrial arrangements are greatly disturbed in all three segments with the mitochondria sometimes running almost parallel to the base of the cells (Fig. 93; double arrows). Numerous dense bodies are observed in all portions of the cytoplasm especially in segment 3 (Fig. 94).

The CNT-cells and the intercalated cells are filled with granular material, and unusual nucleoli are seen (Figs. 95; single arrow). Apically located uncoated vesicles are often absent in these cells (Fig. 95), whereas they are greatly increased in the intercalated cell (Fig. 97; single arrow).

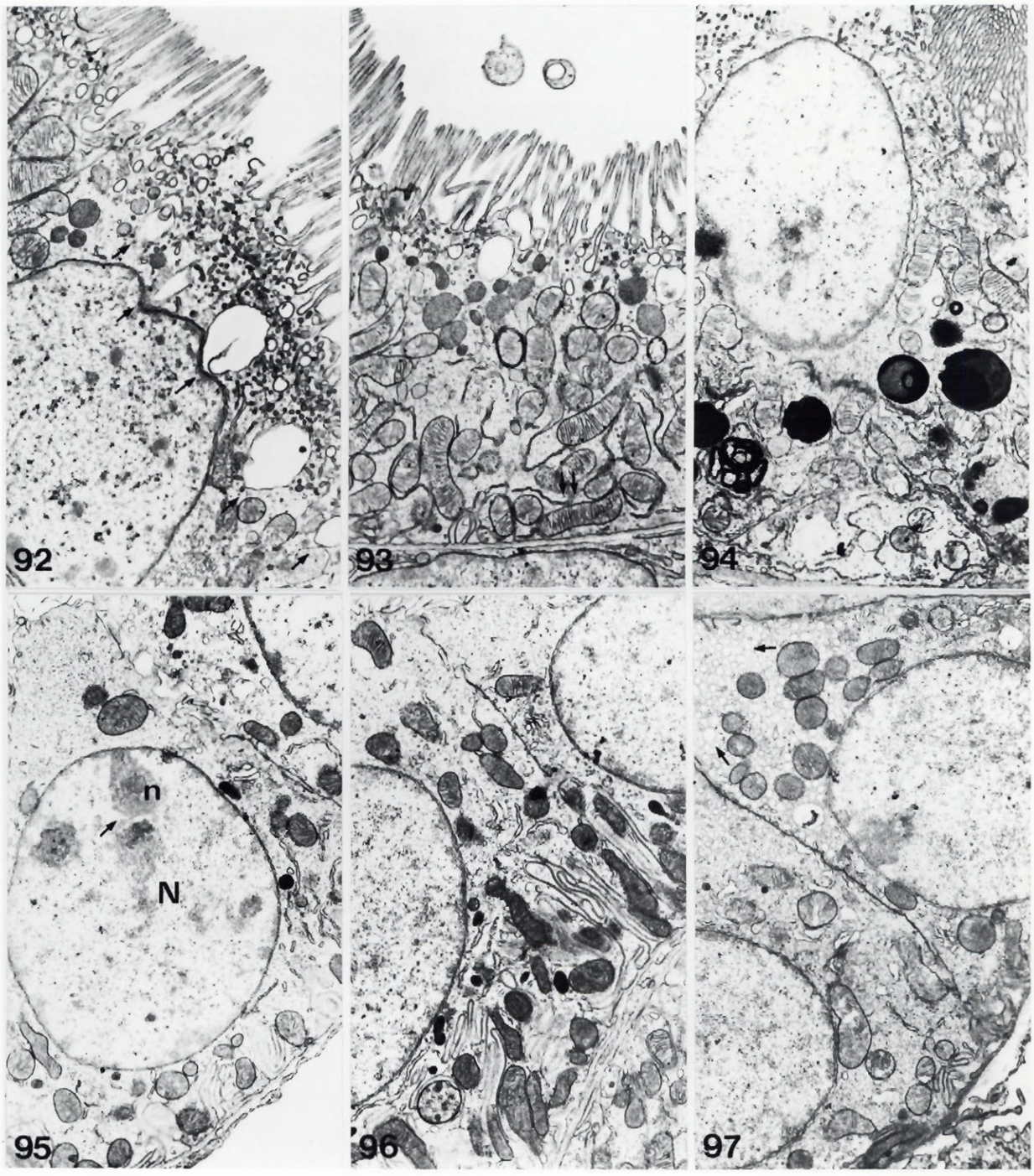

92
93
94
n
N
95
96
97

PLATE 27

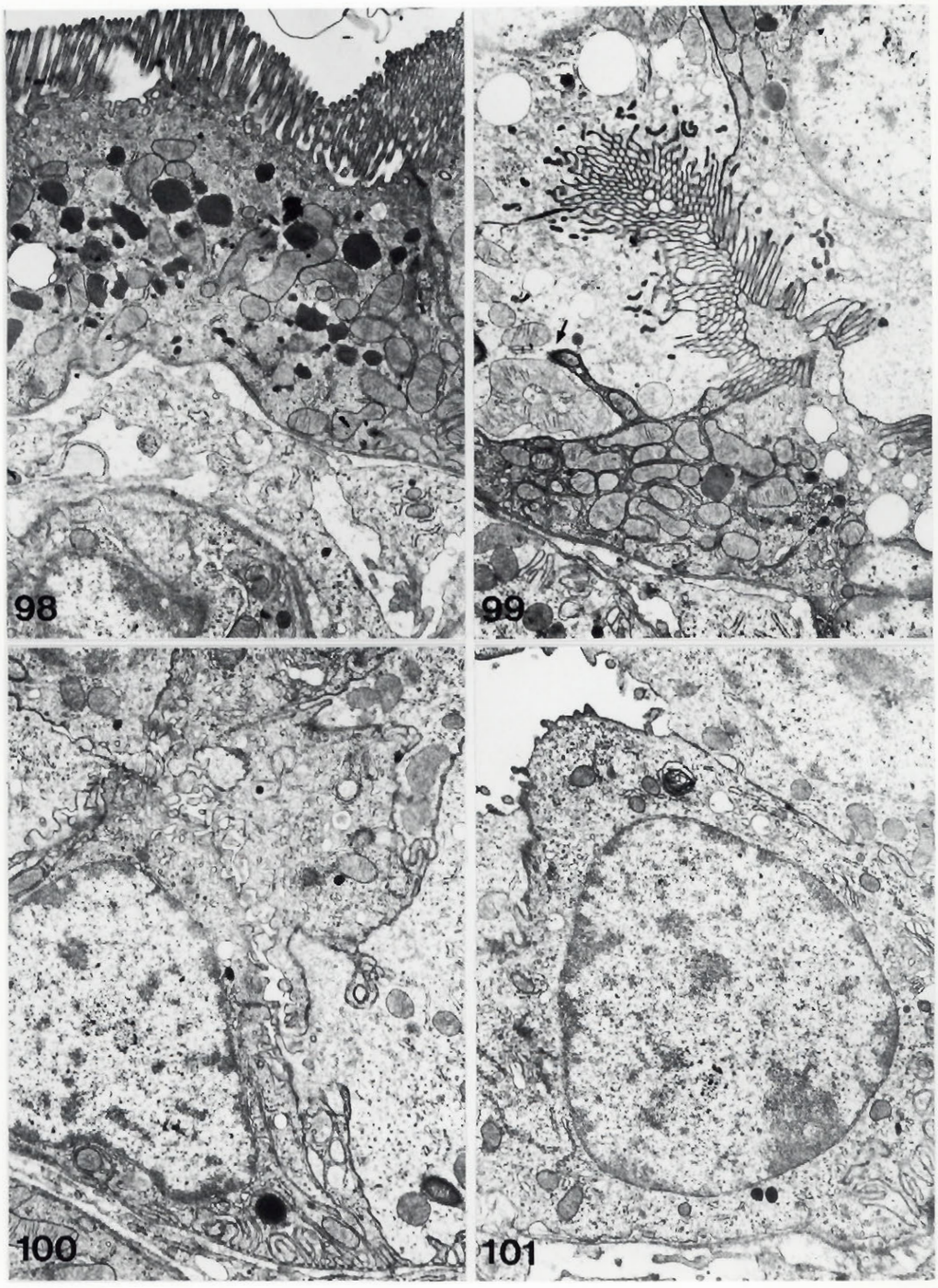
Electron micrographs

Histology of the proximal and distal tubule portions of the nephron of incisor absent ia/ia rats.

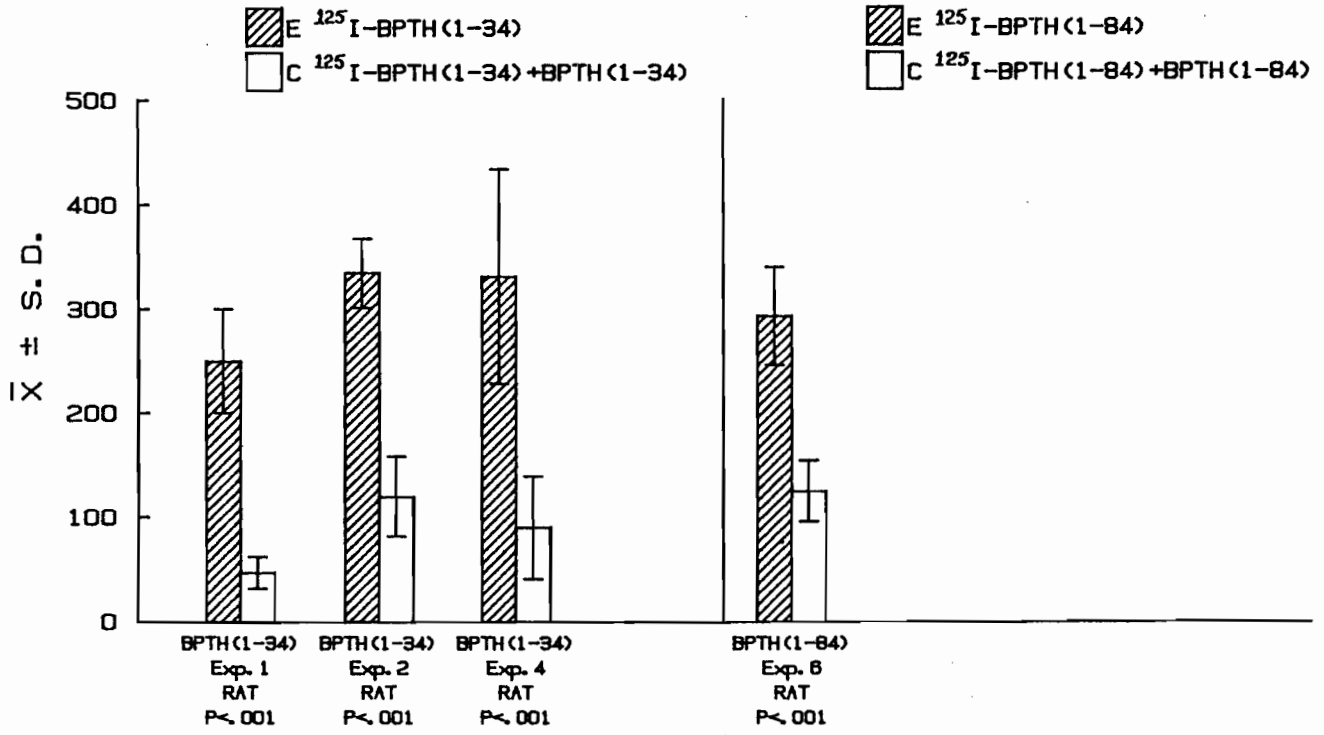
Figures 98-101 Electron micrographs of renal tissue obtained fron incisor absent ia/ia rats.
SCT Exp 5 IA Mutant rat.

In the ia/ia mutant there is a major increase in the amount of dense cytoplasmic bodies in the proximal tubular cells (Fig. 98;arrow) and as in the osteosclerotic oc/oc mice the basolateral membrane infoldings are greatly perturbated. Mitochondria may be short and randomly arranged in a dense cytoplasm, or large in an electron lucent cytoplasm (Fig. 99; single arrow). Many cells have large vacuoles.

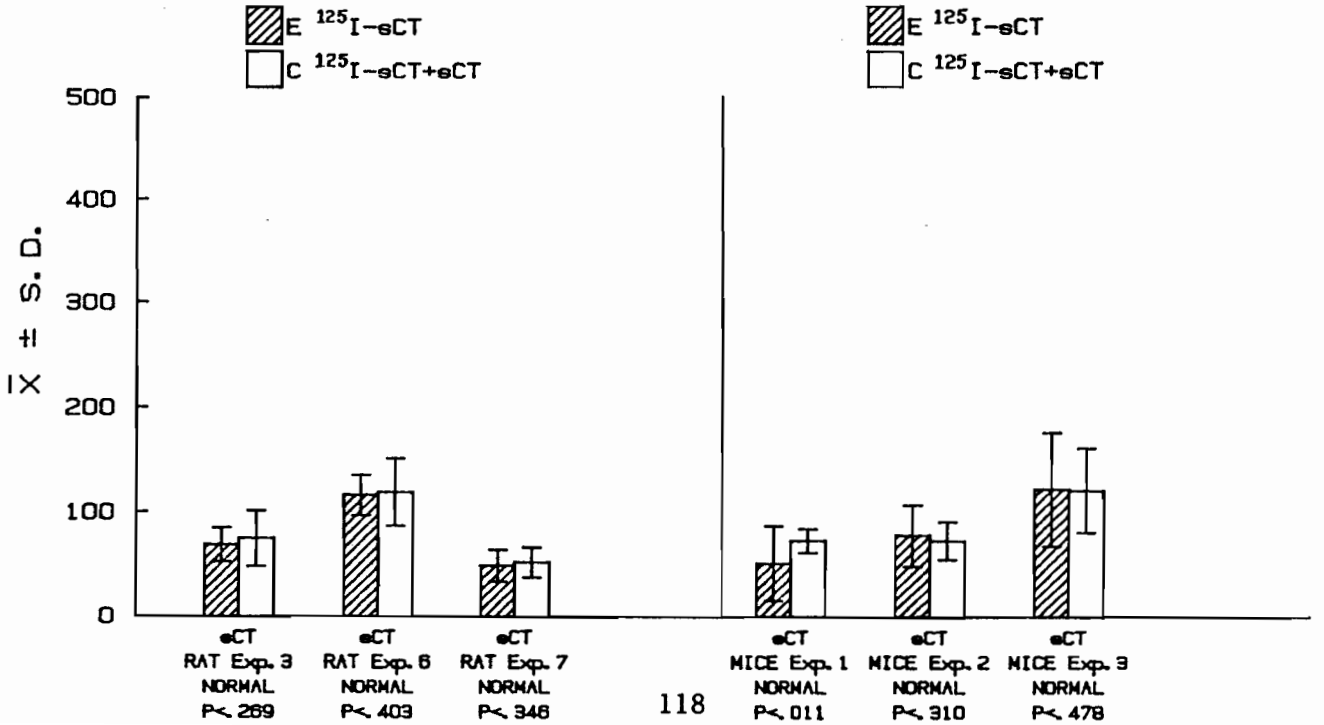
In the distal tubular segment, the amount of mitochondria are very low in the CNT-cells (Fig. 100) and the intercallated cells (Fig. 101). The amount of uncoated vesicles is greatly decreased as well. X7,125.


98
99
100
101

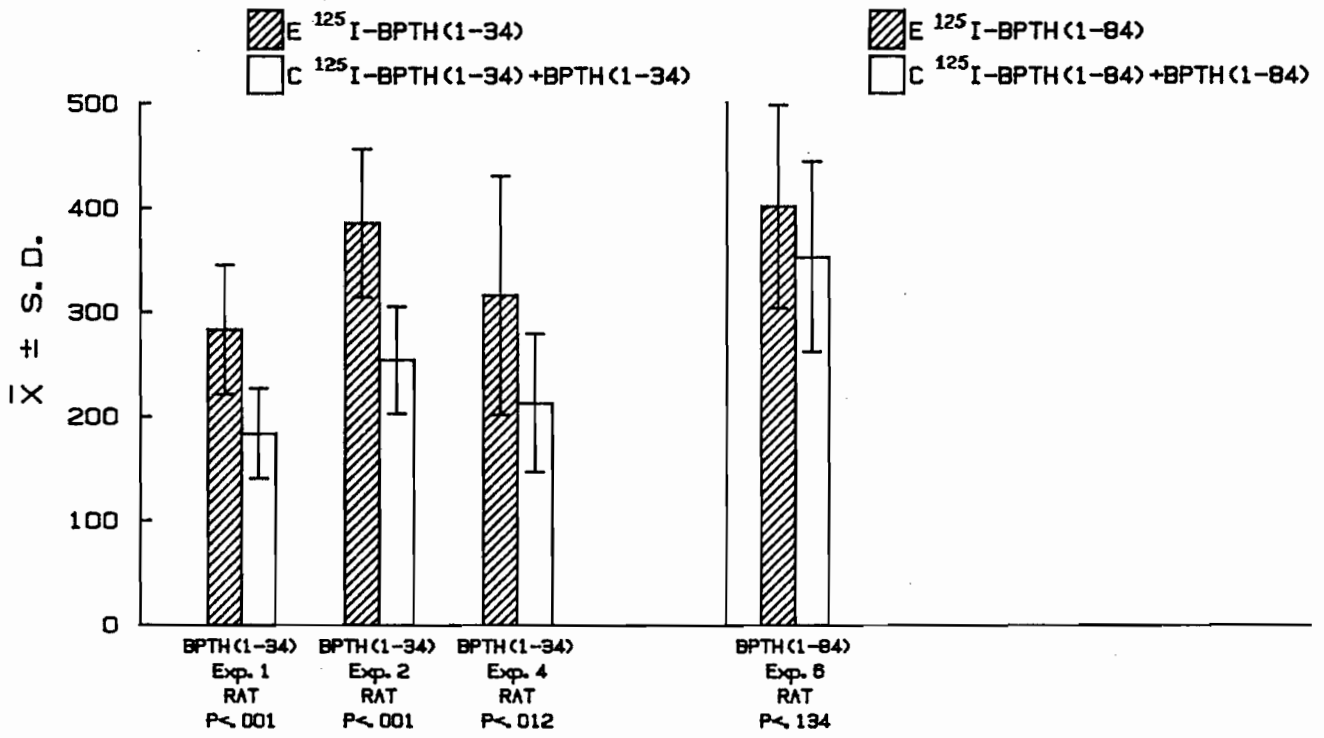
GRAPH 9. **Binding of ^{125}I-bPTH related to glomerular parenchyma in kidney tissue of normal rats.**



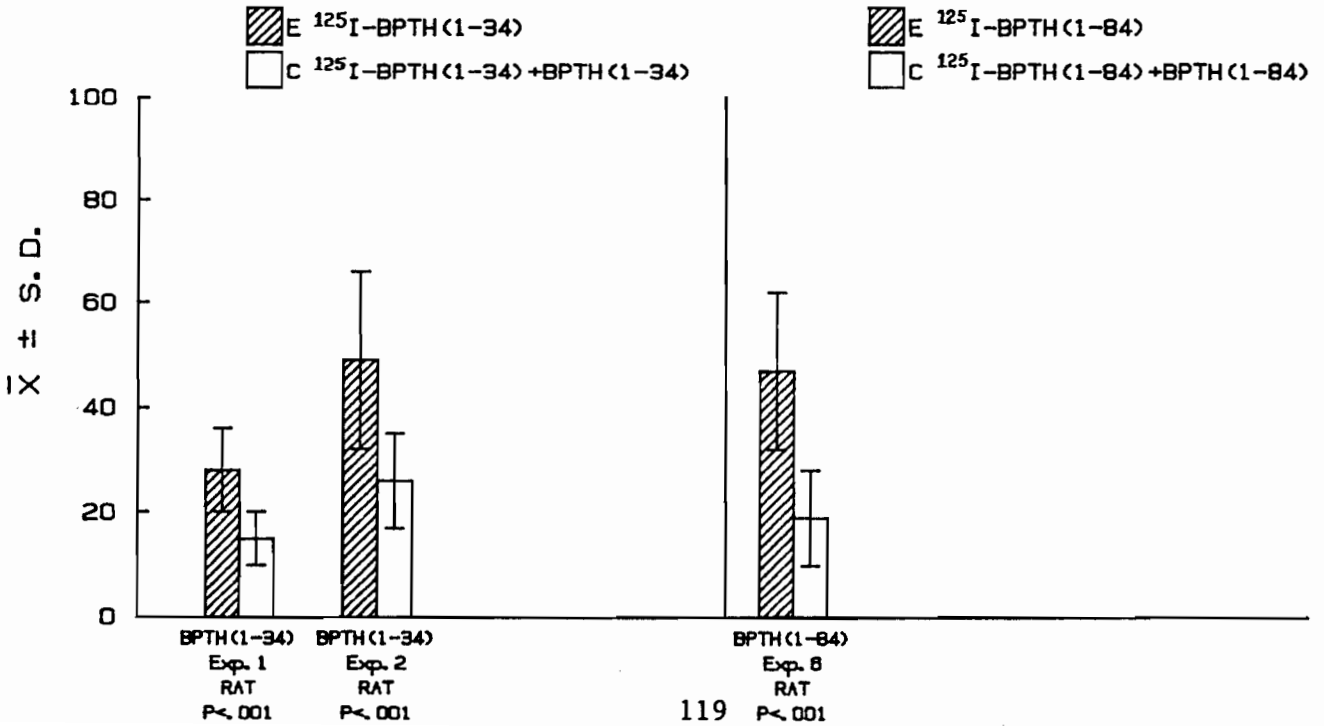
GRAPH 10. **Binding of ^{125}I-sCT related to glomerular parenchyma in kidney tissue of normal rodents.**



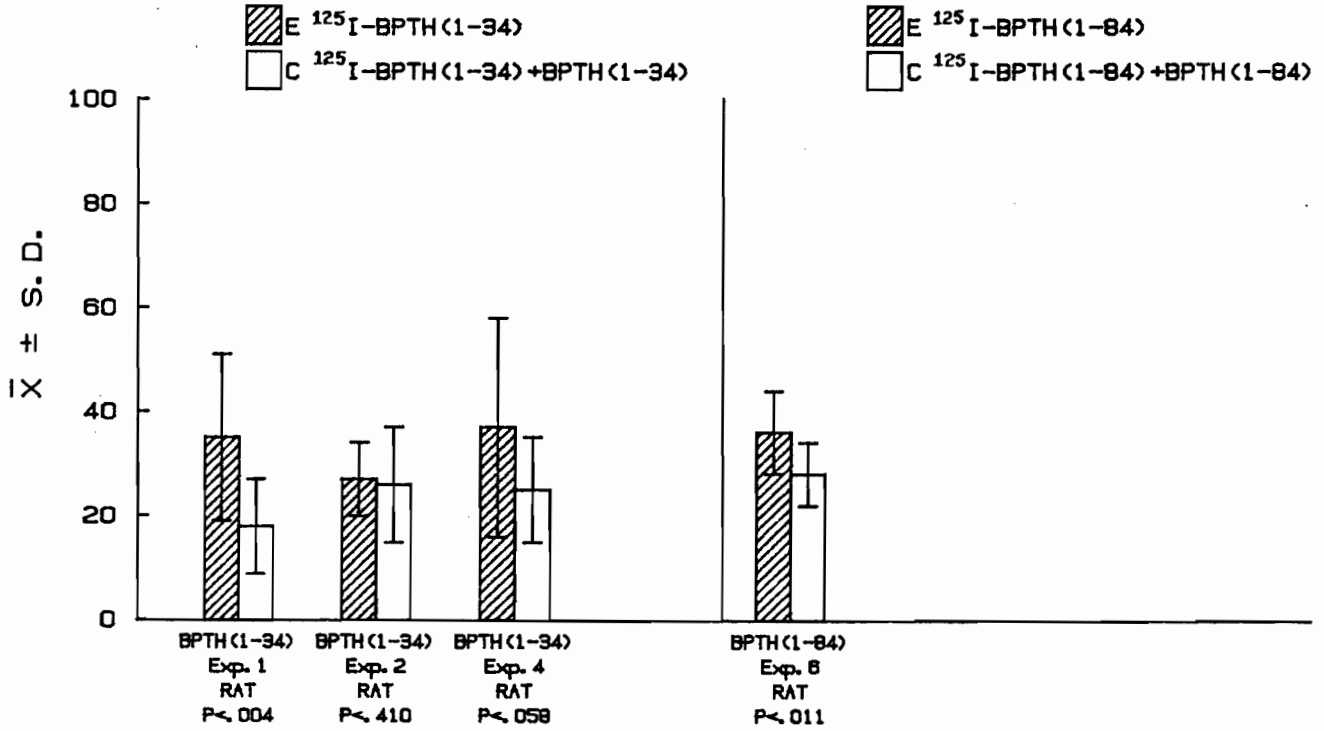
GRAPH 11. Binding of ^{125}I-bPTH to segment 1 of the proximal tubule portion (PTS_1) of the nephron in kidney tissue of normal rats.



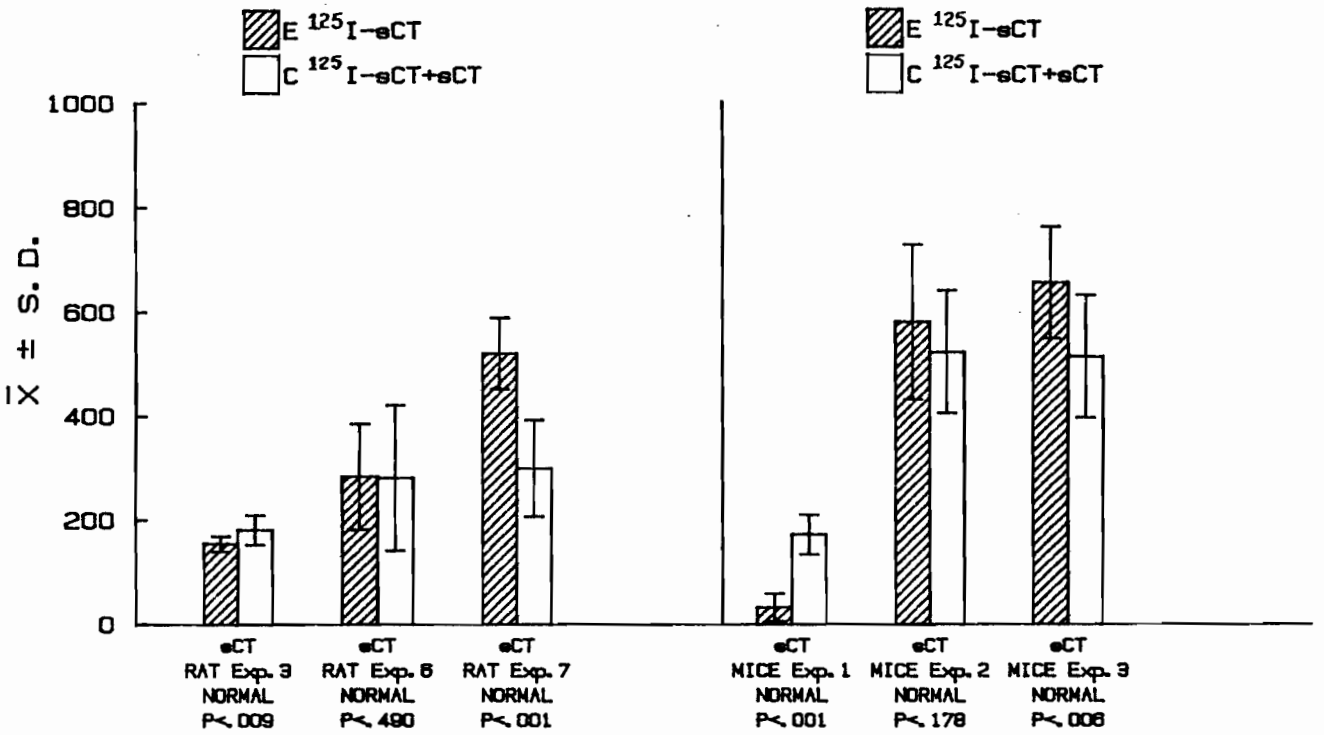
GRAPH 12. Binding of ^{125}I-bPTH to segment 2 of the proximal tubule portion (PTS_2) of the nephron in kidney tissue of normal rats.



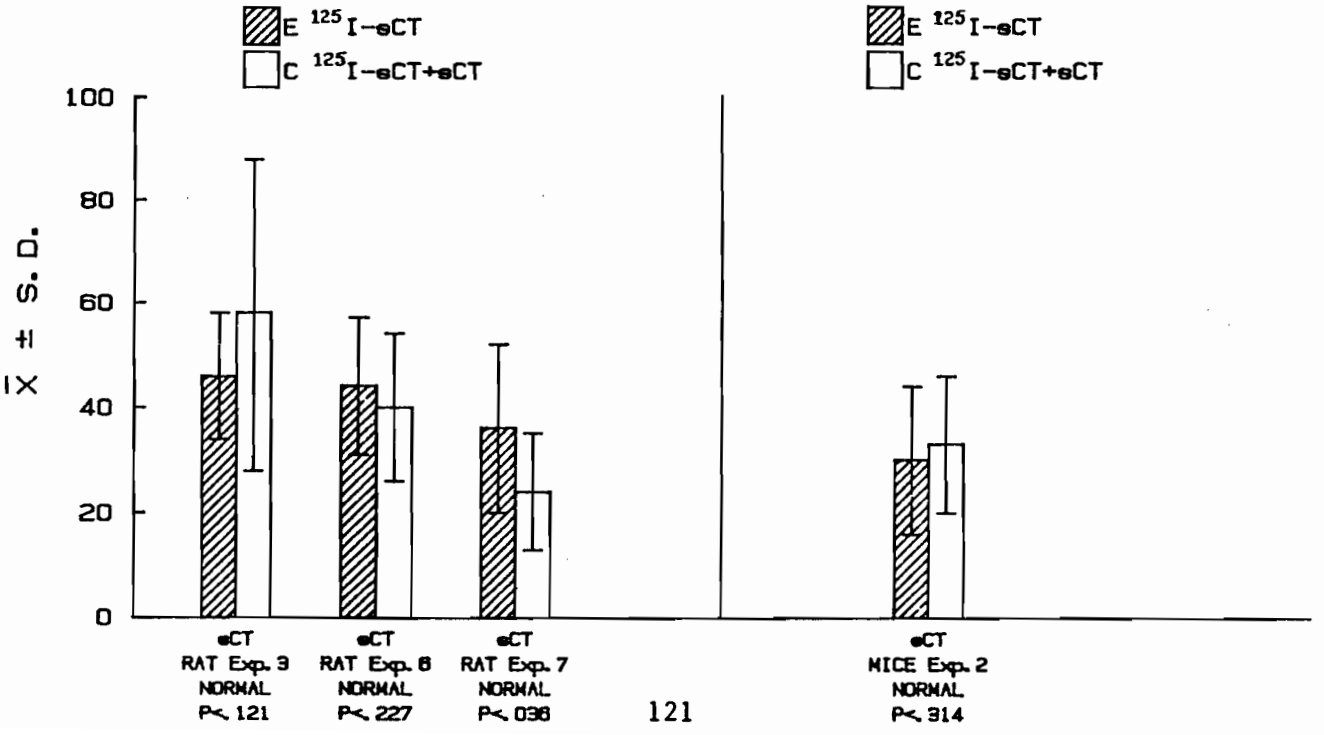
GRAPH 13. Binding of ^{125}I-bPTH to segment 3 of the proximal tubule portion (PTS_3) of the nephron in kidney tissue of normal rats.



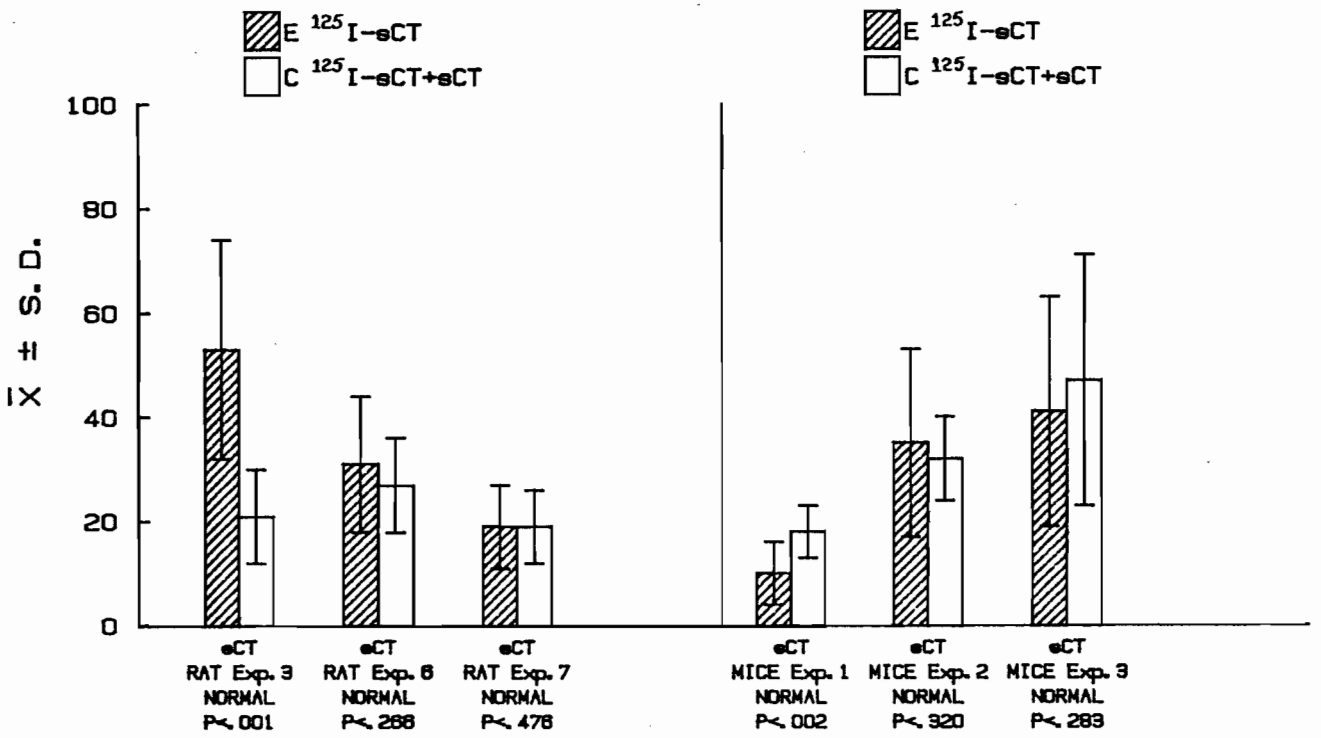
GRAPH 14. Binding of ^{125}I-sCT to segment 1 of the proximal tubule portion (PTS_1) of the nephron in kidney tissue of normal rodents.



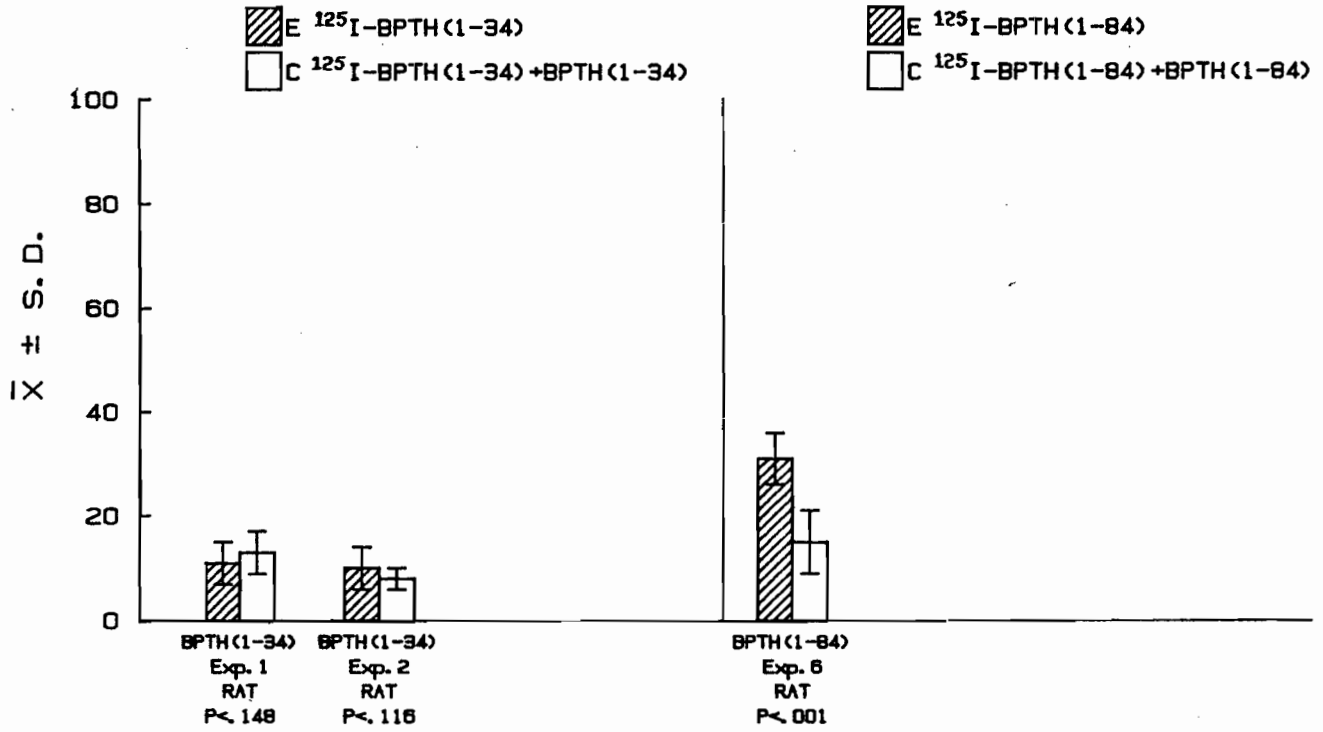
GRAPH 15. Binding of ^{125}I-sCT to segment 2 of the proximal tubule portion (PTS_3) of the nephron in kidney tissue of normal rodents.



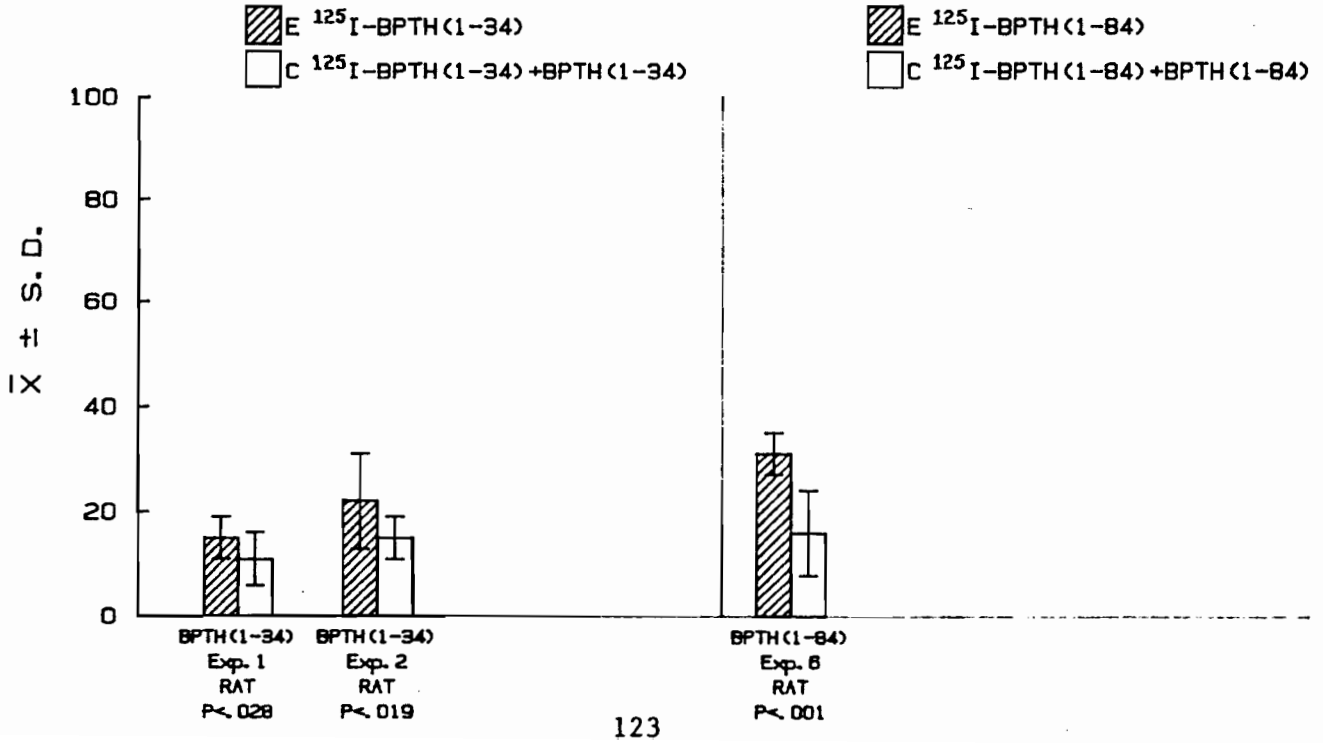
GRAPH 16. Binding of ^{125}I-sCT to segment 3 of the proximal tubule portion (PTS_3) of the nephron in kidney tissue of normal rodents.



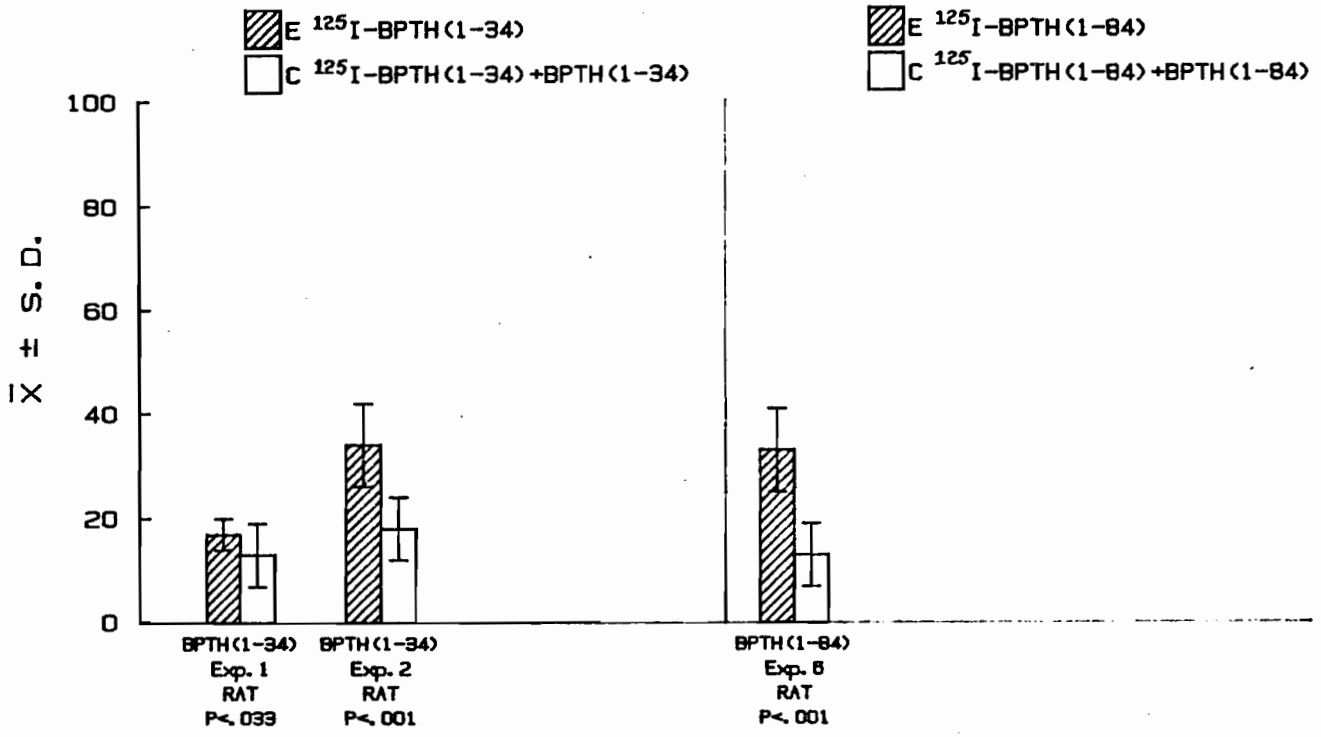
GRAPH 17. Binding of ^{125}I-bPTH to the ascending thick limb of the distal tubule portion of the nephron found in the inner stripe of the outer medula (MAL I_s) in kidney tissue of normal rats.



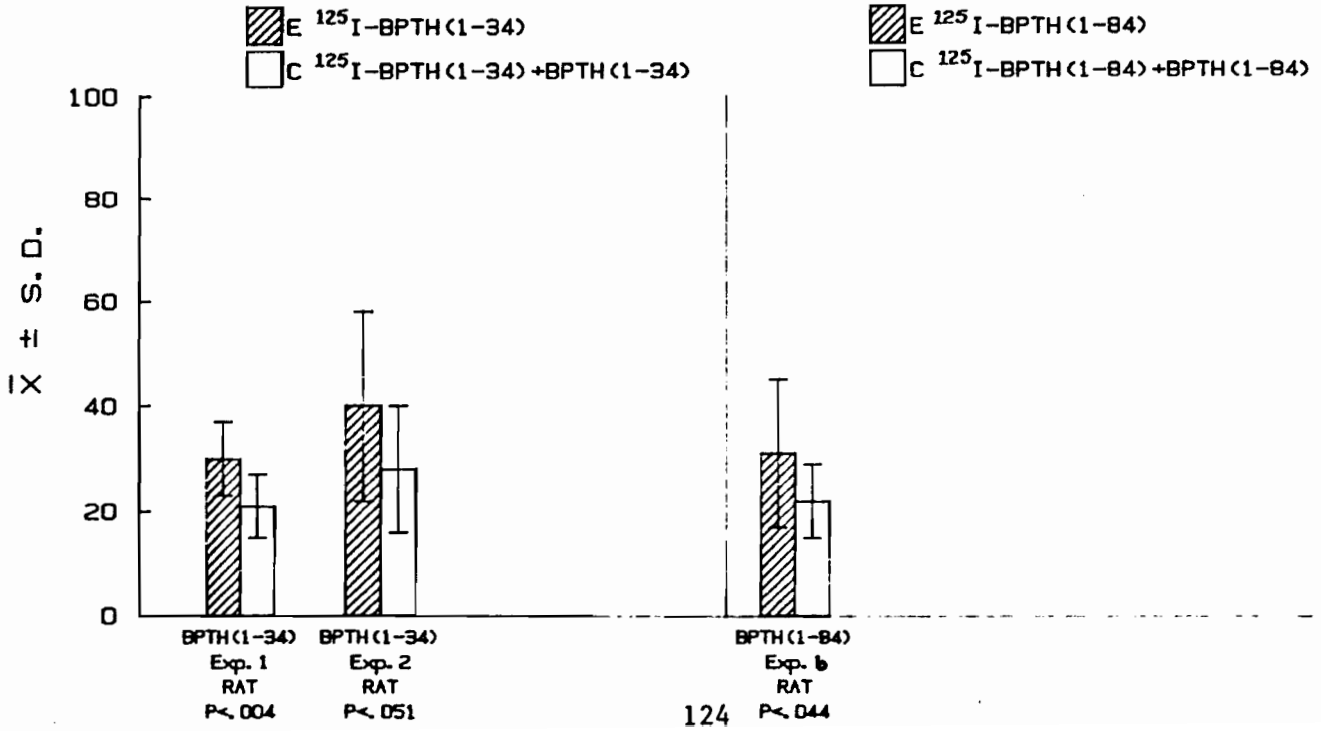
GRAPH 18. Binding of ^{125}I-bPTH to the ascending thick limb of the distal tubule portion of the nephron found in the outer stripe of the outer medula (MAL O_s) in kidney tissue of normal rats.



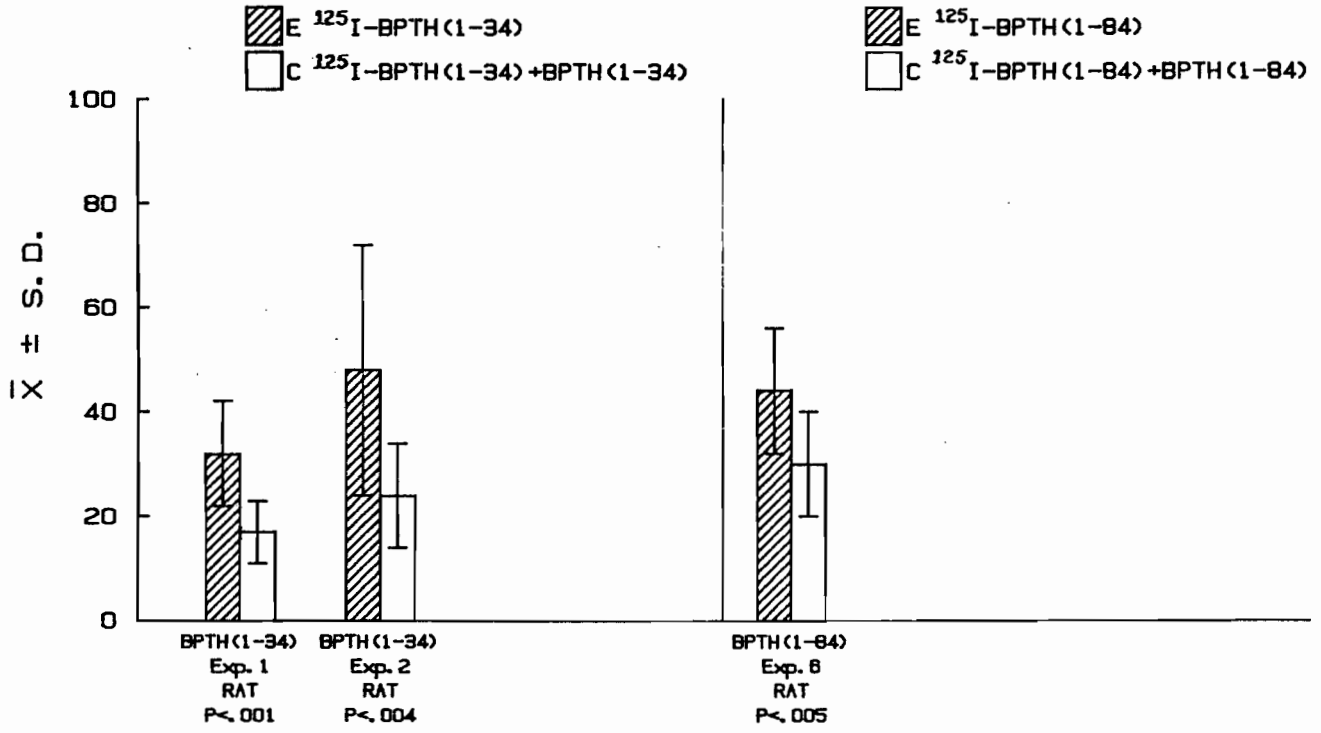
GRAPH 19. Binding of ^{125}I-bPTH to the ascending thick limb of the distal tubule portion of (CAL) the nephron found in the cortex in kidney tissue of normal rats.



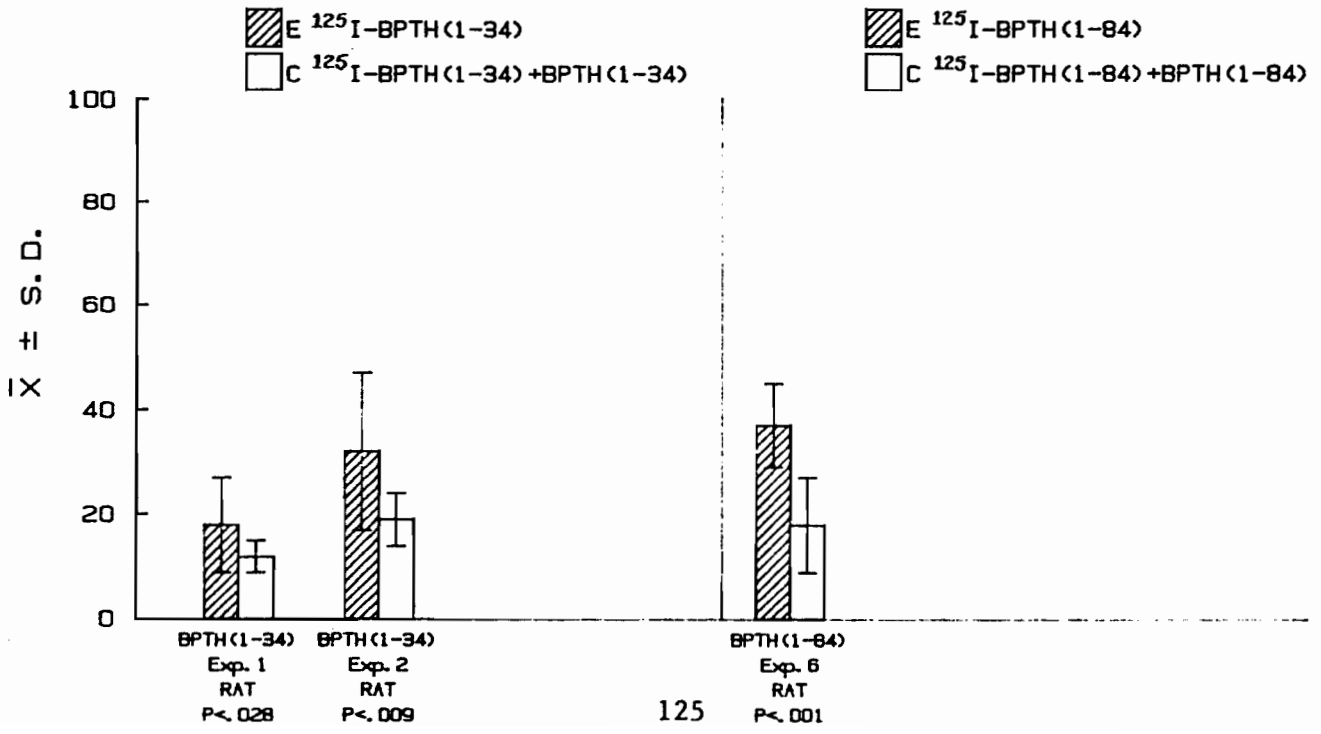
GRAPH 20. Binding of ^{125}I-bPTH to the distal convoluted tubule portion (DCT) of the nephron in kidney tissue of normal rats.



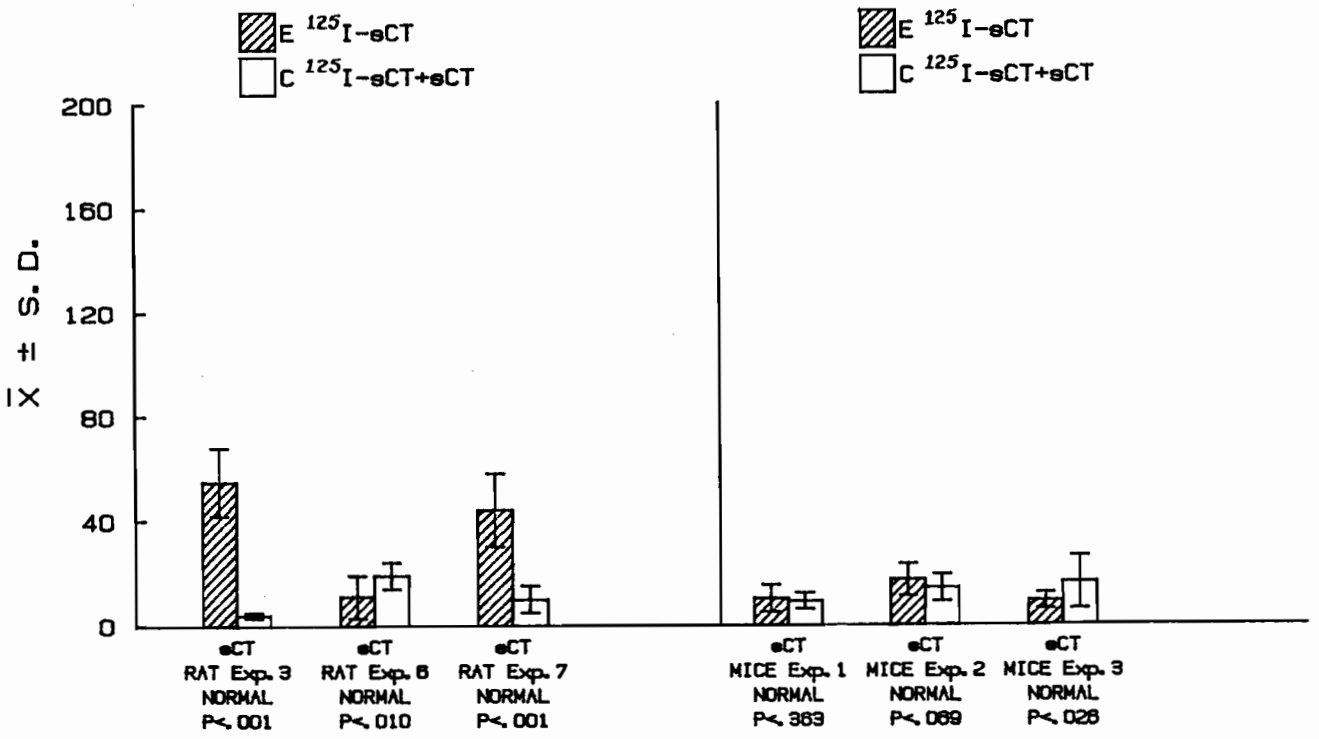
GRAPH 21. Binding of ^{125}I-bPTH to the connecting tubule portion (CNT) of the nephron in kidney tissue of normal rats.



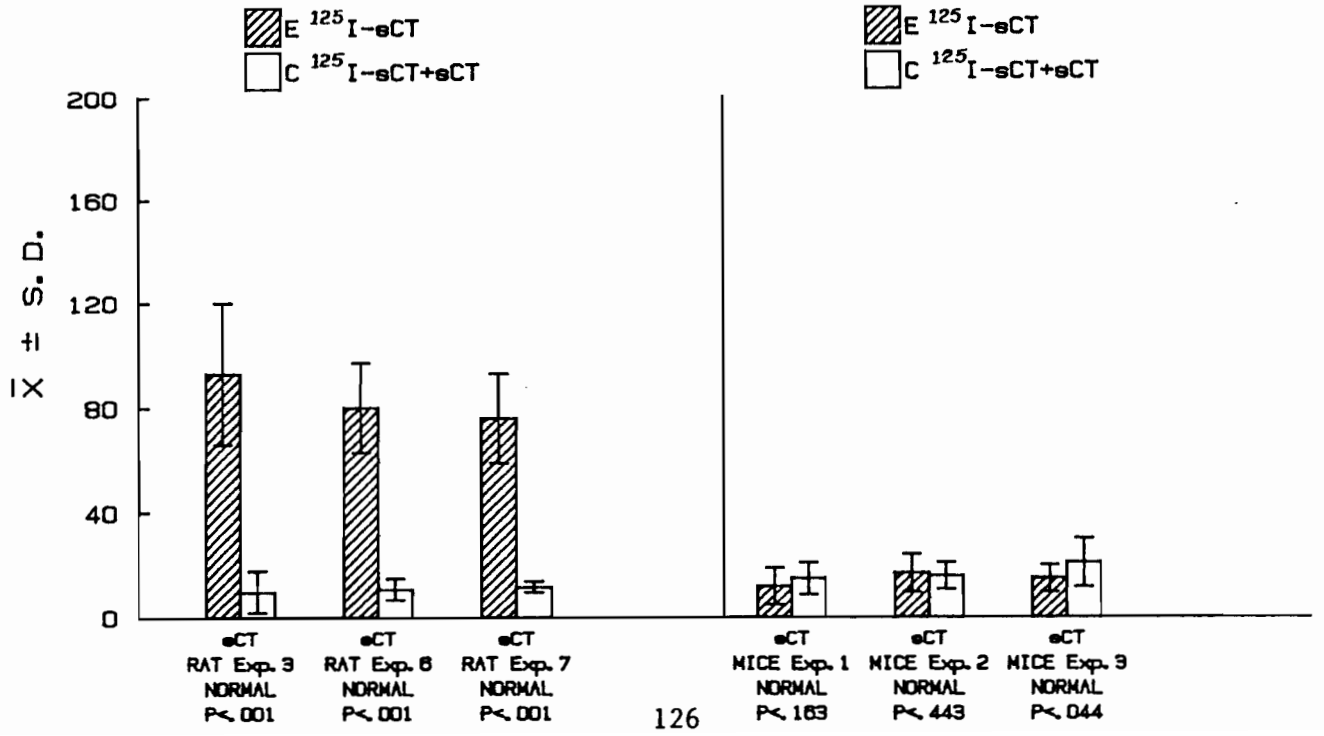
GRAPH 22. Binding of ^{125}bPTH to the arched collecting tubule portion (ACT) of the nephron in kidney tissue of normal rats.



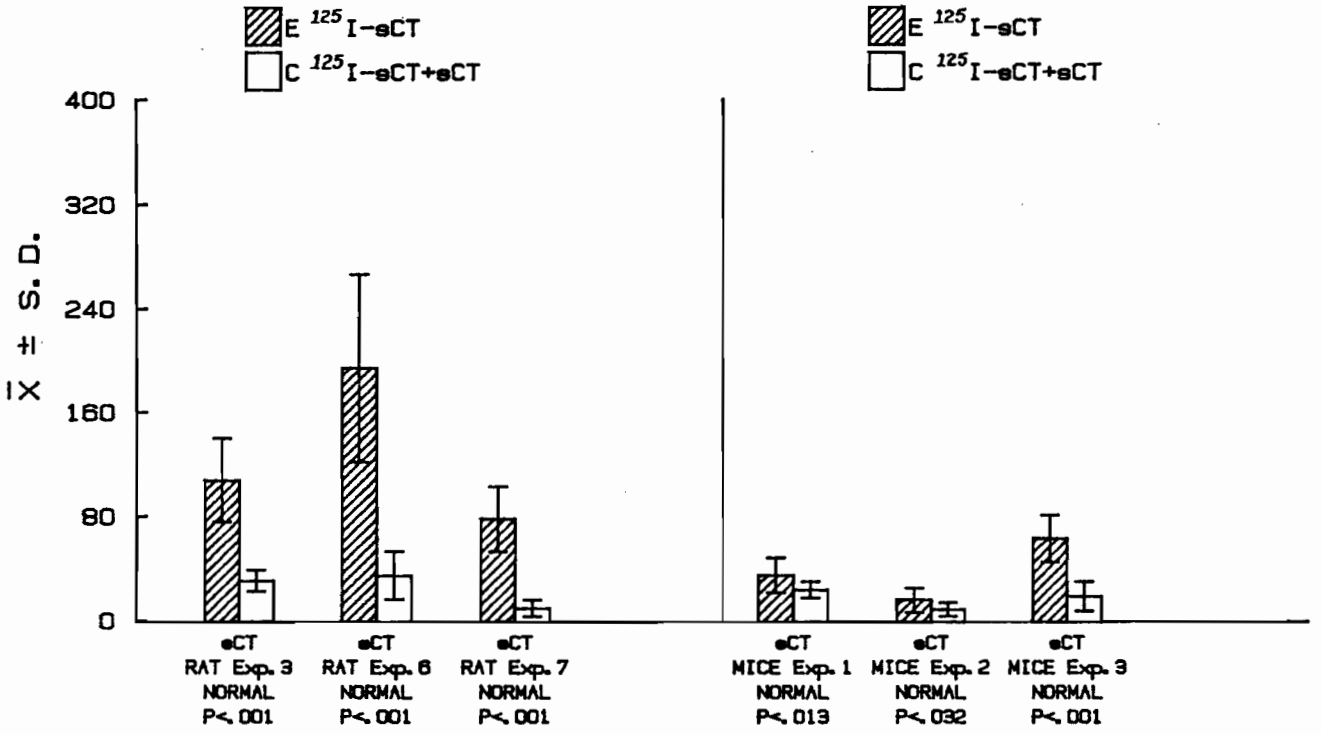
GRAPH 23. Binding of ^{125}I-sCT to the ascending thick limb of the distal tubule portion of the nephron found in the inner stripe of the outer medula (MAL I_s) in kidney tissue of normal rodents.



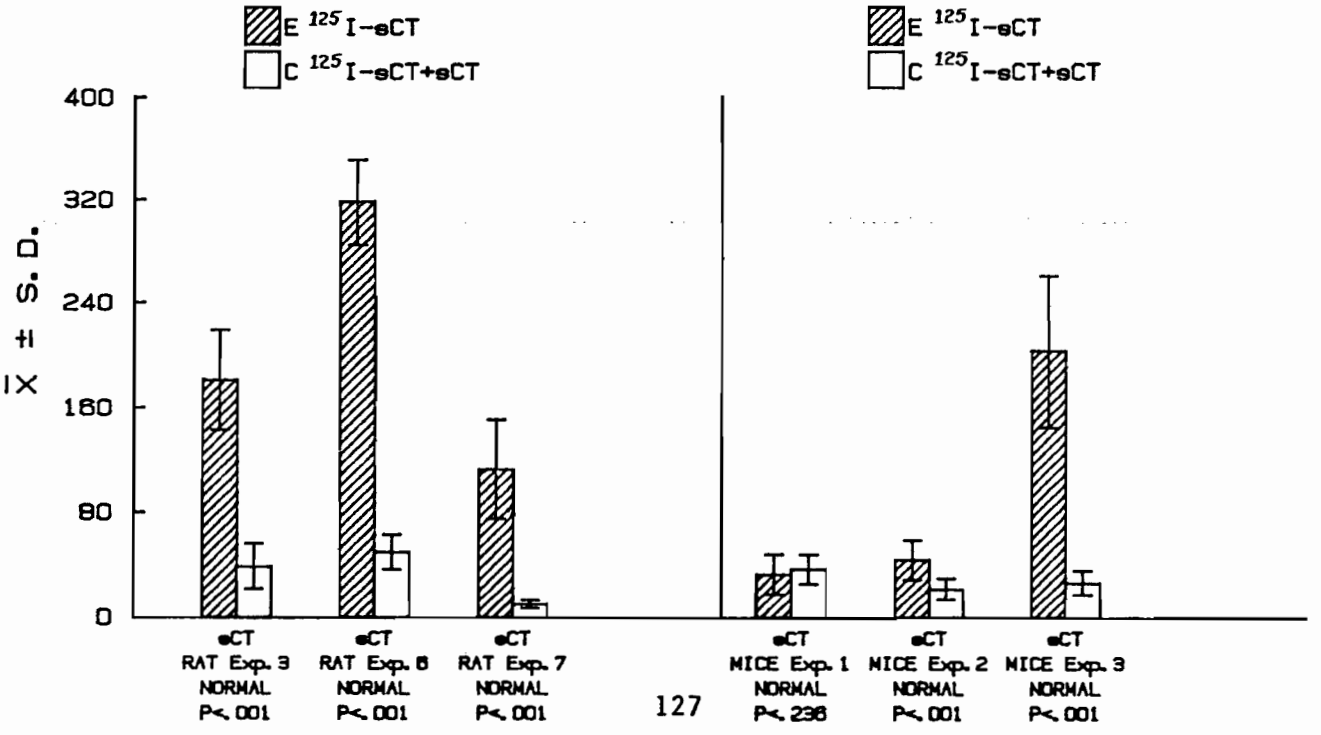
GRAPH 24. Binding of ^{125}I-sCT to the ascending thick limb of the distal tubule portion of the nephron found in the outer stripe of the outer medula (MAL O_s) in kidney tissue of normal rodents.



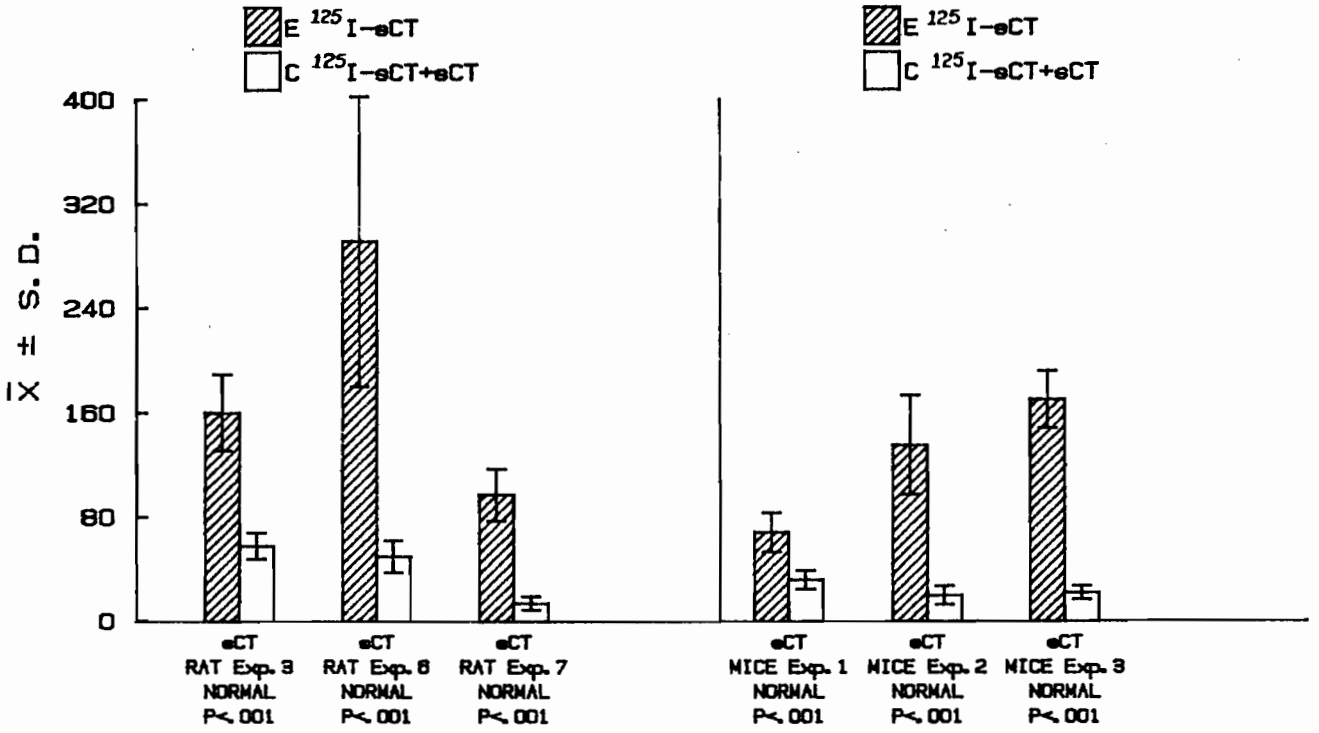
GRAPH 25. Binding of ^{125}I-sCT to the ascending thick limb of the distal tubule portion of the nephron found in the cortex (CAL) in kidney tissue of normal rodents.



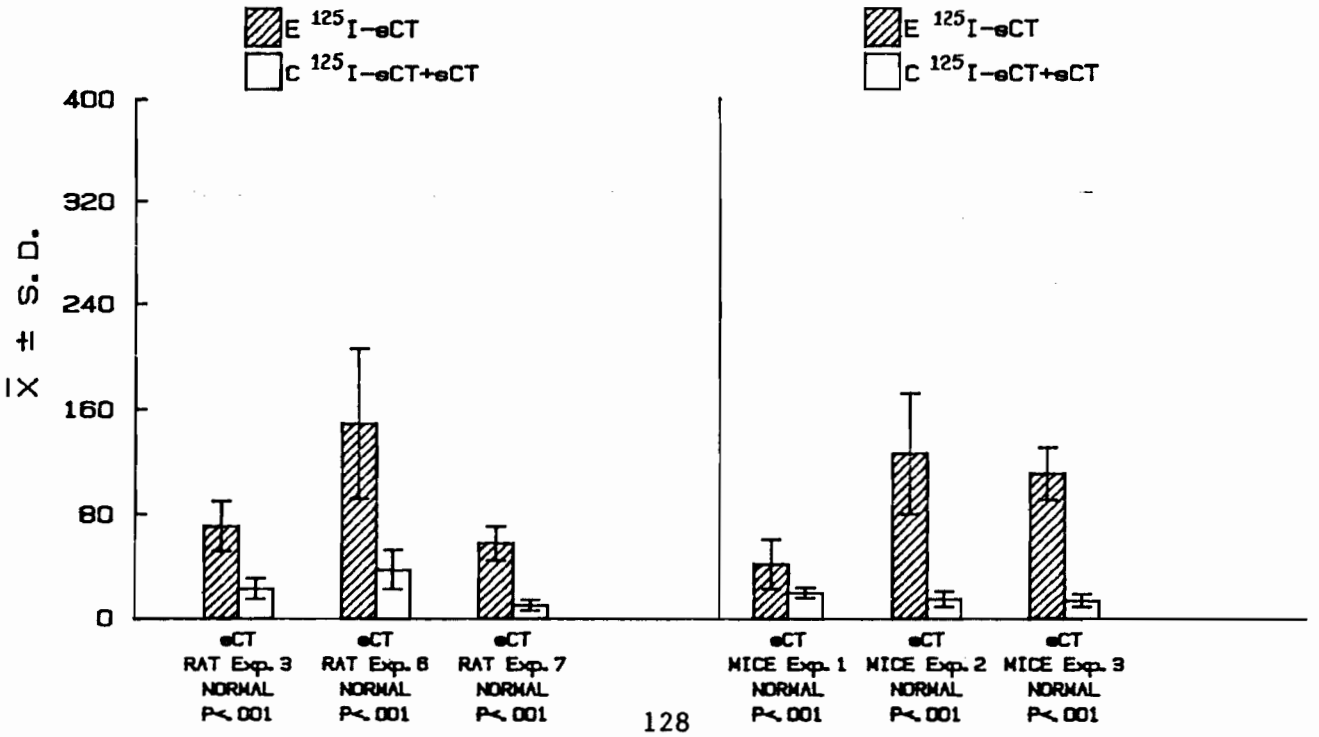
GRAPH 26. Binding of ^{125}I-sCT to the distal convoluted tubule portion (DCT) of the nephron in kidney tissue of normal rodents.



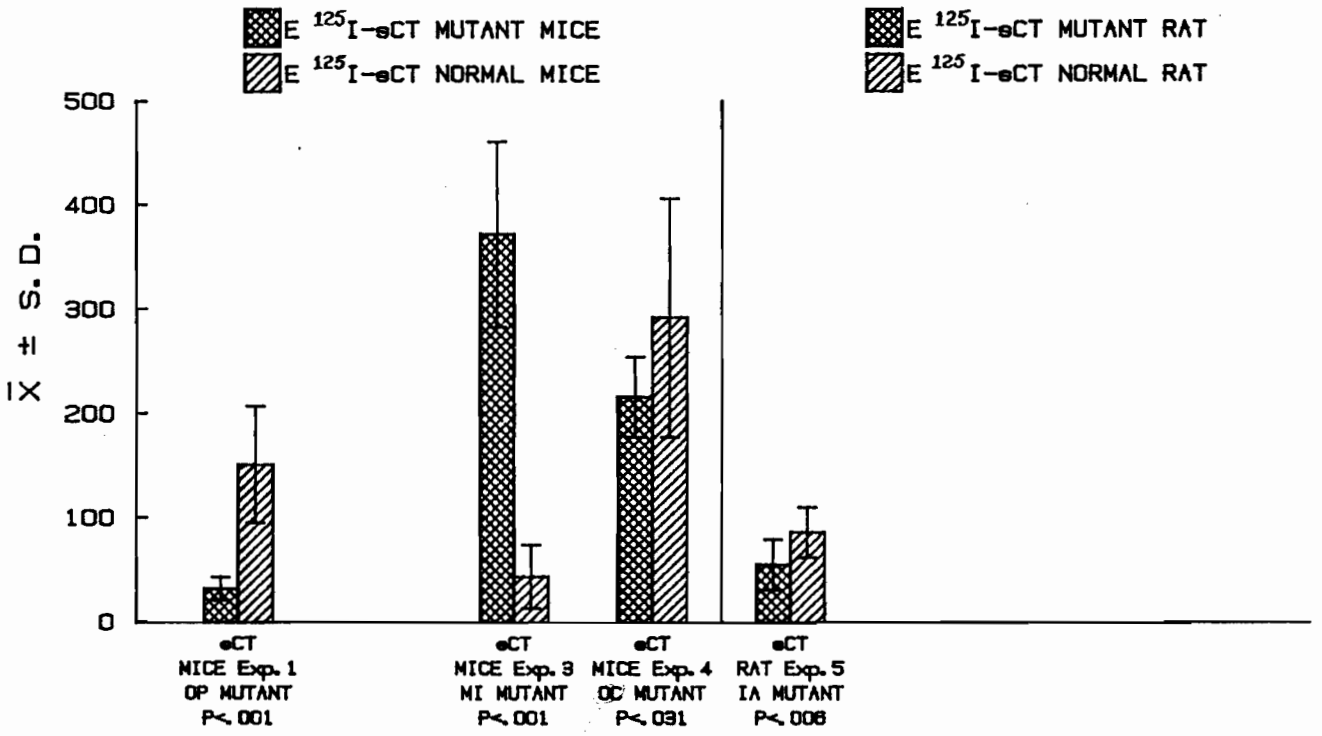
GRAPH 27. Binding of ^{125}I-sCT to the connecting tubule (CNT) of the nephron in the kidney tissue of normal rodents.



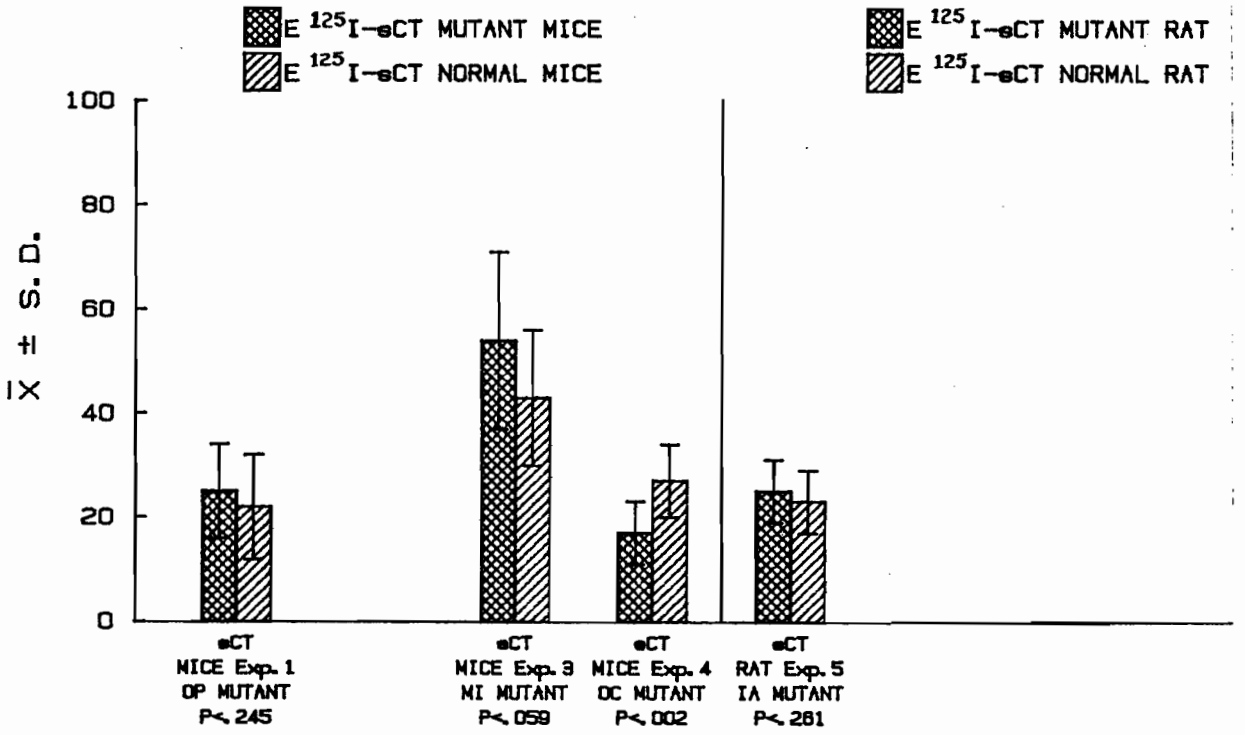
GRAPH 28. Binding of ^{125}I-sCT to the arched collecting tubule portion (ACT) of the nephron in kidney tissue of normal rodents.



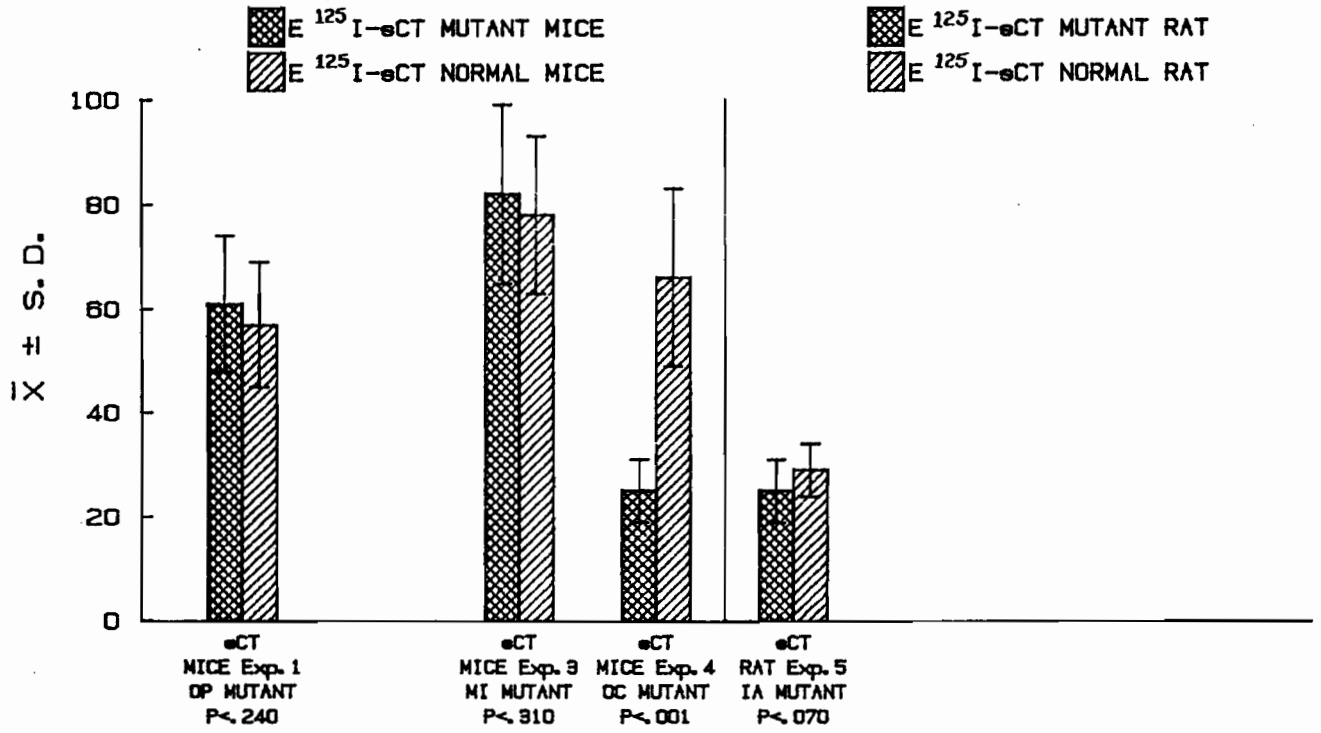
GRAPH 29. **Binding of ^{125}I-sCT to segment 1 of the proximal tubule portion (PTS_1) of the nephron in kidney tissue of osteopetrotic mutant rodents.**



GRAPH 30. Binding of ^{125}I-sCT to the distal convoluted tubule portion (DCT) of the nephron in kidney tissue of osteopetrotic mutant rodents.



GRAPH 31. Binding of ^{125}I-sCT to the connecting tubule portion (CNT) in kidney tissue of osteopetrotic mutant rodents.



GRAPH 32. Binding of ^{125}I-sCT to the arched collecting tubule portion (ACT) of the nephron inkidney tissue of osteopetrotic mutant rodents.

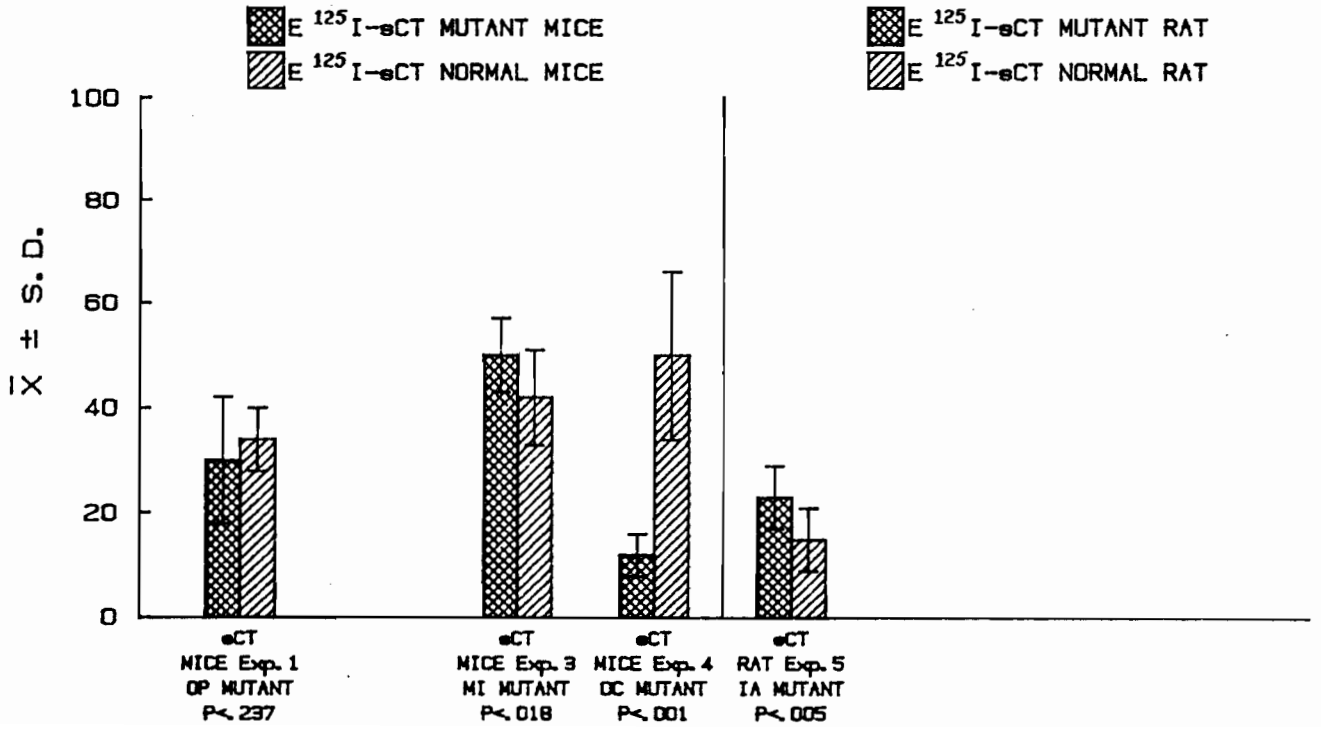


TABLE 5

Competitive inhibition study of ^{125}I-bPTH(1-34) with excess unlabeled bPTH(1-34) and insulin in target tissues of normal rats
BPTH(1-34) Exp 3

	Hepatic Tissue	Renal Tissue					
		RC	PTS_1	PTS_2	DCT	CNT	ACT
^{125}I-bPTH(1-34)							
Mean	**560**	**125**	**535**	**20**	**15**	**23**	**13**
SD	80.4	34.0	153.9	5.0	2.7	10.1	2.8
SEM	20.2	10.7	48.7	1.6	0.8	3.19	0.9
....+ bPTH(1-34)							
Mean	**275**	**36**	**341**	**18**	**16**	**12**	**12**
SD	62.3	9.0	119.4	5.9	4.4	3.6	5.9
SEM	14.6	2.9	37.8	1.9	1.4	1.1	1.9
P Value	**0.001**	**0.001**	**0.003**	**0.180**	**0.274**	**0.003**	**0.285**
....+ insulin							
Mean	**634**	**119**	**473**	**23**	**16**	**21**	**16**
SD	80.5	23.7	170.4	9.8	4.2	8.1	4.7
SEM	25.4	7.5	53.9	3.1	1.3	2.6	1.6
P Value	**0.009**	**0.321**	**0.202**	**0.158**	**0.311**	**0.333**	**0.062**

RC (renal corpuscle); PTS_1 (segment 1 of the proximal tubule); PTS_2 (segment 2 of the proximal tubule); PTS_3 (segment 3 of the proximal tubule); DCT (distal convoluted tubule); CNT (connecting tubule); ACT (arched collecting tubule).

TABLE 6

Competitive inhibition study of ^{125}I-sCT binding
with excess unlabeled sCT and bPTH in target tissues of normal rats
SCT Exp 4

	Osseous Tissue*	Renal Tissue					
		PTS_1	MAL I_s	MAL O_s	CAL	DCT	CNT	ACT
^{125}I-sCT								
Mean	**280**	**417**	**21**	**41**	**61**	**101**	**83**	**59**
SD	99.9	70.6	5.3	8.9	13.6	24.6	24.1	5.8
SEM	20.0	22.3	1.7	2.8	4.3	7.8	7.6	1.8
....+ sCT								
Mean	**43**	**435**	**9**	**11**	**10**	**19**	**27**	**23**
SD	17.6	74.0	1.1	1.6	2.1	3.4	5.2	4.6
SEM	3.5	23.4	0.3	0.5	0.7	1.1	1.6	1.4
P Value	**0.001**	**0.291**	**0.001**	**0.001**	**0.001**	**0.001**	**0.001**	**0.001**
....+ bPTH1-34)								
Mean	**293**	**267**	**25**	**56**	**61**	**110**	**107**	**51**
SD	54.3	115.3	11.8	9.9	12.3	21.2	19.5	17.9
SEM	10.9	36.5	3.7	3.1	3.9	6.7	6.2	5.6
P Value	**0.276**	**0.002**	**0.171**	**0.496**	**0.230**	**0.196**	**0.018**	**0.088**

PTS_1 (segment 1 of the proximal tubule); MAL I_s (medullary thick ascending limb in the inner stripe); MAL O_s (medullary thick ascending limb in the outer stripe); CAL (cortical thick ascending limb); DCT (distal convoluted tubule); CNT (connecting tubule); ACT (arched collecting tubule)

* Mean silver grains per osteoclast profile.

TABLE 7

Competitive inhibition study of ^{125}I-sCT binding
with excess unlabeled sCT and bPTH in target tissues of phenotypically normal mice
SCT Exp 2 OP mice

	Osseous Tissue*	Renal Tissue		
		PTS_1	CNT	ACT
^{125}I-sCT				
Mean	**61**	**98**	**75**	**70**
SD	12.6	104.3	23.0	19.9
SEM	2.5	33.0	7.3	6.3
....+ sCT				
Mean	**11**	**331**	**20**	**14**
SD	4.0	63.3	5.8	4.4
SEM	0.8	20.1	1.8	1.4
P Value	**0.001**	**0.001**	**0.001**	**0.001**
....+ bPTH(1-34)				
Mean	**67**	**78**	**71**	**54**
SD	16.8	25.4	17.3	15.3
SEM	3.4	8.0	5.4	4.8
P value	**0.076**	**0.287**	**0.321**	**0.027**

PTS_1 (segment 1 of the proximal tubule); CNT (connecting tubule); ACT (arched collecting duct).
* Mean silver grains per osteoclast profile.

TABLE 8

Competitive inhibition study of ^{125}I-sCT binding
with excess unlabeled sCT and insulin in target tissues of normal rats
SCT Exp 7

	Hepatic Tissue	Osseous Tissue*	Renal Tissue					
			MAL I_s	MAL O_s	CAL	DCT	CNT	ACT
^{125}I-sCT								
Mean	340	141	44	76	78	113	96	58
SD	47.9	23.3	13.7	17.3	25.1	38.4	19.7	12.9
SEM	12.4	4.7	4.3	5.5	7.9	12.2	6.2	4.1
....+ sCT								
Mean	185	13	10	12	10	14	10	
SD	18.9	5.0	4.9	2.4	3.4	5.0	4.2	
SEM	4.9	1.0	1.6	0.7	1.1	1.6	1.3	
P Value	**0.001**	**0.001**	**0.001**	**0.001**	**0.001**	**0.001**	**0.001**	
....+ insulin								
Mean	510	151	41	71	80	87	125	82
SD	96.2	35.9	21.5	23.1	17.4	36.2	35.6	34
SEM	24.8	7.2	6.8	7.3	5.5	11.4	11.3	11
P Value	**0.001**	**0.134**	**0.380**	**0.288**	**0.436**	**0.070**	**0.020**	**0.024**

MAL I_s (medulary thick ascending limb in the inner stripe); MAL O_s (medullary thick ascending limb in the outer stripe);
CAL (cortical thick ascending limb); DCT (distal convoluted tubule); CNT (connecting tubule);
ACT (arched collecting tubule)
* Mean silver grains per osteoclast profile.

TABLE 9

Binding of ^{125}I-sCT in target tissues in normal male and female rats
SCT Exp 7

	Hepatic Tissue	Osseous Tissue*	Renal Tissue						
			PTS_1	MAL I_s	MAL O_s	CAL	DCT	CNT	ACT
Male									
Mean	**340**	**141**	**519**	**44**	**76**	**78**	**113**	**96**	**58**
SD	47.9	23.3	68.0	13.7	17.3	25.1	38.4	19.7	12.9
SEM	12.4	4.7	21.5	4.3	5.5	7.9	12.2	6.2	4.1
Female									
Mean	**394**	**150**	**514**	**41**	**73**	**99**	**85**	**97**	**72**
SD	62.0	35.6	87.4	13.4	21.7	22.0	20.7	30.5	23.7
SEM	16.0	7.1	27.6	4.2	6.9	6.9	6.6	9.6	7.5
P Value	**0.007**	**0.162**	**0.453**	**0.337**	**0.373**	**0.030**	**0.031**	**0.487**	**0.061**

PTS_1 (segment 1 of the proximal tubule); MAL I_s (medulary thick ascending limb in the inner stripe); MAL O_s (medullary thick ascending limb in the outer stripe);
CAL (cortical thick ascending limb); DCT (distal convoluted tubule); CNT (connecting tubule);
ACT (arched collecting tubule)
* Mean silver grains per osteoclast profile.

PART-IV

DISCUSSION

Osteogenesis

Osteogenesis involves the elaboration of the matrix as well as the deposition of minerals in the matrix secreted by the osteoblasts. Local cells are involved in this process. Much attention has been payed to the osteoblast and to collagen secretion (Leblond and Weinstock, '71). A variety of non-collagenous proteins, among which glycoproteins, have been extracted from the osseous matrix. It is becoming increasingly evident that these extra-cellular components are not only products for structural support, but important regulatory signals for cellular migration, proliferation, and differentiation that produce the precisely organized functional bone tissue (see review in Newman, '80).

There are two major hypothesis on how de novo mineralization is induced during calcification (see review in Wuthier, '82). The first hypothesis claims that mineralization is initiated by a process referred to as heterogeneous nucleation of collagen (Glimcher, '59; Newman, '80), either alone, or in combination with any of several closely associated connective tissue proteins. The second hypothesis is a more directly cell mediated process in which mitochondria (Shapiro and Greenspan, '69; Martin and Matthews, '70; Brighton and Hunt, '78) and matrix vesicles (Anderson, 69; Thyberg and Friberg, 70; Bonucci, 71) are involved.

Deposition of collagen may play a role in the induction of mineralization however, other factors definitively play a role as well. as collagen deposited at other sites in the body is not mineralized. Consequently, the nature of the factors exclusive to the osseous tissue which would stimulate mineralization is an important issue.

On the other hand, the bone matrix itself may play a role in the resorption process. Changes in the type of extracellular matrix during differentiation suggest that these matrices may control cell phenotype and activity. Thus morphogenesis is accompanied by rapid and dramatic changes in components of the extracellular matrix.

However, the control of biological mineralization is most likely the result of complex interplay between stimulatory and inhibitory factors (see review in Newman, '80). This may involve various organelle systems and compartments, intracellular and extracellular processes, organic matrix and inorganic ions in solution as well as those in the solid phase. This is reflected by reports which indicate that in conditions where the concentration of electrolytes is perturbed, osteopathies are observed (Bell, '67; Avioli and Teitelbaum, '76).

Bone Modeling and Bone Remodeling

Osteolysis, which consists of the breakdown and removal of mineralized bone is a dominant process during skeletal growth and serves a major role in **modeling** the osseous mass. Bone resorption is much reduced during adulthood and consists of a much slower process refer to as **remodeling**, a phenomena whereby bone is consistantly replaced by de novo bone. Additionally, demineralization of osseous tissue also occurs for the purpose of calcium homeostasis. The resorption of osseous tissue implies both mineral dissolution and removal of bone matrix. Calcium homeostasis need not involve matrix destruction or hydroxyapatite crystal dissolution, because a portion of the skeletal calcium is amorphous in form and readily available (Bringhurst and Potts, '79).

During skeletal growth, bone formation prevails; during adulthood, skeletal osteolysis prevails and may to some extent cause a condition in the elderly known as osteoporosis.

Cinematographic studies of osteoclasts in tissue culture have presented convincing evidence that osteoclasts participate actively in bone resorption (Gaillard, '55). The osteocyte has been proposed as a possible active agent in the bone resorbing process (Belanger and Migicovsky, '63). Mononuclear cells, as well as osteoblasts have additionally been considered as playing secondary roles (Heersche, '78; Rodan and Martin, '81).

The bone matrix itself may play a role in bone resorption. Osteoclasts are chemotactically attracted to exposed bone. The osteoclasts appear to recognize some component of the mesenchyme of the presumptive skeletal site as a signal to invade.

Osteogenesis appears to be regulated by PTH whereas osteolysis is regulated by both PTH and CT. Although much literature already describes PTH and CT involvement in osteogenesis and osteolysis, there is still a great deal of uncertainty about which cells are acted upon by which hormones, as well as about what role these two hormones play in the modulation of these two processes. Besides their direct involvement with osseous tissue, both of these hormones are acting at other target sites. Since it is likely that the physico-chemical environment is a major factor in mineralization, it seems important not to neglect the binding at other sites where PTH and CT may play a role in osseous tissue metabolism and pathology.

PTH and CT Binding in Normal Rodents :Functional Considerations

Binding to hepatic tissue

It has long been believed that the liver is not the site of any major interaction with calcitonin, however, the organ is known to be involved in PTH metabolism.

Parathyroid hormone

Circulating PTH exists in more than one form (Silverman and Yalow, '73). The molecular forms that have received attention are intact PTH(1-84), the bioactive NH_2-terminal, and the presumably inactive COOH-terminal fragments.

Using an *in vivo* assay, competitive specific binding for the NH_2-terminal end has been demonstrated. It is accompanied by a dose-dependent generation of cAMP indicating a high specificity and a presumably high affinity hormone-receptor interaction (Bergeron et al., 81).

In the *in vivo* study, the radioautographic reaction was quantitated over liver parenchyma devoid of obvious Kupffer cells. At the electron microscope level, ^{125}I-bPTH reaction has been localized to hepatic and sinusoidal cells. Binding to the hepatocytes was inhibited maximally by excess related hormone, however, binding to the sinusoidal endothelial cells was inhibited to a much lesser extent. This indicates the presence of at least 2 groups of receptors. The first group is low capacity presumably high affinity receptors localized to the cell surface of the hepatocytes. The second group is higher capacity, presumably lower affinity receptors localized to sinusoidal cells.

Role of the competitive binding of ^{125}I-bPTH to hepatocytes

PTH in the liver causes an increase in the fractional coefficient for calcium influx (Chausmer et al., '72), activates the adenylate cyclase system (Canterbury et al., '73; Bergeron et al., '81), and increases gluconeogenesis (Hruska et al., '79). PTH play a role in the liver transformation of vitamin D_3 into 25-hydroxycholecalciferol (Ponchon and DeLuca, '69). Alternatively, PTH may interfere with the synthesis or release of albumin, one of the major calcium binding proteins (Bringhurst, and Potts, '79).

Role of the binding of ^{125}I-bPTH to sinusoidal endothelial cells

The sinusoidal cells were found in the present study to bind and internalize ^{125}I-bPTH. The walls of sinusoids are loosely arranged, therefore they do not interfere with the passage of such molecules. Thus, it is unlikely that internalization of the labeled hormone occurs for purpose of translocation from the sinusoidal lumen to the space of Disse. A similar interaction of the sinusoidal cell with other hormones, including insulin (Bergeron et al., '79), has been observed.

Although intact, as well as the COOH-terminal fragments have been reported to be extracted by the kidneys (Martin et al., '79), it is the liver that removes the greatest portion of the circulating hormone. Moreover, PTH has a longer half life in hepatectomized animals, and exposure of the hormone to liver homogenates results in inactivation of the bioactivity (Fang and Tashjian, '72). Consequently, a most probable function of the sinusoidal binding would be that of inactivation of a certain fraction of the injected hormone.

Role of the binding of ^{125}I-bPTH(1-84) to Kupffer cells

When intact ^{125}I-bPTH(1-84) was injected a great number of silver grains were related to Kupffer cells. The binding of labeled bPTH(1-84) is decreased by co-injection of unlabeled bPTH(1-84). The very low amount of reaction associated with these cells when bPTH(1-34) was injected suggests a sequence-specific interaction for bPTH at these loci.

Whereas the kidney extracts intact hormone, together with COOH-, as well as NH_2-terminal fragments, the liver selectively extracts intact hormone (Martin et al., '76). The preferential binding of bPTH(1-84) to the Kupffer cells suggests a role for these cells in bPTH cleavage into the circulating fragments. Although these fragments may be liberated by the parathyroid glands under certain conditions and to a lesser extent may be generated in the kidney (D'Amour et al., '79), the liver appears to be the main site of parathyroid hormone cleavage into the active (NH_2-terminal) and the presumably inactive fragments. The first clevage occurs between position 33-34 (Segre et al., '74; Martin et al., '76; Segre et al., '78). The role played by the Kupffer cells in intact hormone cleavage is strongly supported by the observation that isolated Kupffer cells incubated in vitro, metabolized PTH to fragments that are chemically and immunochemically identical to those found in the plasma in vivo (Segree et al., '81).

Calcitonin

Similar to ^{125}I-bPTH, ^{125}I-sCT binds to hepatocytes and sinusoidal cells. The pattern of silver grain distribution, and the relative ability of excess unlabeled hormone to competitively inhibit binding of the labeled hormone, are identical.

Role of competitive binding of ^{125}I-sCT to hepatocytes

In rats, CT causes a significant decrease in serum calcium while increasing the liver calcium concentration (Chausmer et al., '65; Yamaguchi et al., '75). Moreover, calcitonin treatment brings about a significant decrease in the liver plasma membrane Ca-ATPase (a membrane enzyme involved in the energy dependent pathway for the cellular efflux of calcium) (Yamaguchi, '79). The hypothesis that the action of calcitonin on the liver does not involve cAMP production (Canterbury et al., '74) has been challenged, as in vitro, sCT increases cAMP production (Bergeron; personal communication).

In view of the biological responses to CT, the liver would be expected to be a site of competitive specific binding. In experiments previously reported, ^{125}I-sCT binding was inhibited by simultaneous injection of unlabeled related hormone, but not by PTH, insulin and ACTH (de Luise et al., '70). Thus, the observation that sCT binds to low capacity presumably high affinity, specific binding sites in the liver in the present study is not surprising.

Role of binding of ^{125}I-sCT to the sinusoidal cells

As already described for bPTH binding, the most likely function of hormone binding to the sinusoidal endothelial cells is removal and inactivation of a certain portion of the circulating sCT.

Observations on the binding of ^{125}I-sCT in the liver of mice

In mice, the binding of ^{125}I-sCT to liver is substantial. Comparison of the reactions obtained in the Experimental animals, with those obtained in the Control animals, showed a non-statistically significant difference indicating non-specific binding.

However, the binding in mouse liver tissue (Fig. 12) appears identical in intensity and in distribution to that in the normal rat (Fig. 10). Moreover, gamma counter estimation cpm/gr of the radioactivity content in dissected liver tissue obtained from one of the experiments (SCT Exp 2 Normal mice; Experimental 5.36 X 10^6 cpm/g; and Control 1.70 X 10^6 cpm/gr), indicated that the ratio of tissue radioactivity content did not correspond to the quantitative radioautographic counts recorded in Graph 2. The possibility of technical error during tissue preparation argues for the presence of competitive binding for ^{125}I-sCT in mouse liver tissue.

Binding to skeletal tissue

Parathyroid hormone and calcitonin are both known for their involvement with bone metabolism. Whereas PTH has been reported to enhance bone formation as well as bone resorption (Talmage, '67) there seems to be a general agreement on the exclusive role of calcitonin as an inhibitor of osteoclastic bone resorption (Holtrop et al., '74).

Parathyroid Hormone

In skeletal preparations, *in vitro*, PTH activates the adenylate cyclase system (Aurbach and Chase, '70). Specific, high affinity binding for ^{125}I-bPTH has been demonstrated over osteoblasts and presumed osteoprogenitors using the *in vitro* competitive binding approach in 18 day cultured embryonic chick calvaria (Silve et al., '82) and in osteoblast-like, osteosarcoma cell cultures (Rizzoli et al., '83). Using an immunohistochemical approach, bPTH binding of a relatively higher density, when compared with that of other bone cells, was

observed over osteoclasts (Rao et al., '83). Finally, it has been suggested that osteocytic-osteolysis is PTH mediated (Belanger, '65).

In this thesis, qualitative and quantitative analysis of the ^{125}I-bPTH reaction distribution in light microscope radioautographs indicated competitive-specific binding to osteoblasts, but not to osteoclasts.

In electron microscope preparations, ^{125}I-bPTH binds to three morphologically distinct cells: osteoblasts, sinusoidal cells, and to bone connective tissue mononuclear cells. The connective tissue cell is recognized by the irregular nucleus and by an abundance of Golgi elements. The binding of ^{125}I-PTH was inhibited maximally over osteoblasts, and to a lower extent over the sinusoidal cells in radioautographs obtained from the Control animals. From the present data, it must be concluded that the well documented PTH effect on osteoclastic function is not the direct result of a PTH-interaction with osteoclasts.

Significance of the absence of ^{125}I-bPTH binding to osteoclasts

The evidence for PTH stimulation of bone resorption appears unshakable. There is a large amount of structural and physiological evidence that PTH stimulates osteoclastic function. Most of the evidence comes from systems studying the release of ^{45}Calcium or ^{3}H-hydroxyproline preincorporated into bone. The studies all agree that this is a PTH-induced increase in the release of the two markers (King et al., '75).

The release of isotopic markers is accompanied by an increase in osteoclast number (Weisbrode et al., '74; Baron and Vignery, '81) as well as by an increase in the ruffled border and clear zone of osteoclasts upon PTH-treatment (Holtrop et al., '74; King et al., '75).

PTH promotes an increase in cytoplasmic content, as well as in extracellular release of acid phosphatase from osteoclasts (Vaes, '68; Lucht and Maunsbach, '73). It induces an increase in osteoclast cytoplasmic RNA, as measured by ^{3}H-Uridine incorporation. The increase in RNA synthesis is accompanied by a corresponding stimulation of protein and mucoprotein synthesis (Bingham et al., '69).

Although it is well established that PTH brings about an increase in osteoclast number, an increase in the resorption function has been disputed. Cameron and co-workers reported no PTH induced changes in the ultrastructure of osteoclasts (Cameron et al., '67), and although Holtrop and co-workers reported on PTH-induced increase in ruffled border occurence, Holtrop also pointed out the inability of investigators to demonstrate consistent PTH-induced changes in this cell (Holtrop and King, 77). In TPTX rats, PTH-treatment shows that osteoclast ultrastructure is similar to that in the control animals (Weisbrode et al., '74). It has been theorized that all of the osteoclast alterations in structure or in function may be secondary to a direct action on osteoblasts, which hypothetically could promote the removal of the osteoblastic barrier by inducing changes in osteoblasts which would uncover the matrix making them available for osteoclast action (Rodan and Martin, '81).

The sequence of events occuring in bone after PTH-induced generation of cAMP is not understood, and there is still some uncertainty about the nature of the cell, or cells, which generate this factor. It is believed that the net bone resorbing activity is the result of at least 2 different responses with regard to time. An early response occuring within minutes (Talmage, '67) which could result from PTH enhancement of the

osteocytic-osteolytic process (Belanger, '65), and a more delayed osteoclast-osteolytic response most probably accompanied by a less pronounced osteoblast induced bone synthesis (see review in Raisz and Kream, '83).

The increase in osteoclastic function appears far too late to account for an early response to PTH, which may be crucial for calcium homeostasis. It has been proposed that the rapid component of the PTH-induced hypercalcemic response involves the movement of calcium already in solution coming from a bone fluid compartment (Talmage et al., '75) not involving demineralization. As the PTH-calcium increase is not accompanied by a parallel increase in serum phosphate (see review in Parsons, '79) it indicates that no hydroxyapatite crystal dissolution needs to take place to generate the PTH induced hypercalcemia.

Alternatives to a direct action of PTH on osteoclasts

Osteoclast precursors are of hematogeneous origin (see review in Marks, '83). Although PTH may not be acting on mature osteoclasts, an increase in number could well result from activation of osteoclast precursors. Results of the present work, using electron microscope radioautographs, show that ^{125}I-bPTH binds to connective tissue mononuclear cells (Figs 33, 34). Based on morphological criteria, i.e. the irregular contour of the nucleus, the abundance of free ribosomes, and the presence of a complex Golgi apparatus associated with dense granules believed to be primary lysosomes, the cells resemble pre-osteoclasts as already described (Scott, '67). Similar cells have been described in fetal rat calvaria (Rifkin et al., '80).

It was proposed that bone resorption is a process that requires

the activity of two cell types. Resorption would be initiated by the osteoclast demineralizing areas of bone and degrading the collagen matrix, followed by phagocytosis of exposed collagen fibrils by mononuclear fibroblast-like or monocyte derived cells (Heersche, '78).

The above hypothesis rests on the belief that bone resorption involves 2 different cellular mechanisms, **exocytosis** which involves the secretion of lytic enzymes at the bone-cell interface, and **endocytosis**, which involves the uptake and digestion of material in the cytoplasm. Peroxidase, thorium dioxide, ferritin, and lanthanum are endocytosed by the osteoclast (see reviews in Gothlin, and Ericsson, '76; Holtrop and King, '77) which suggests that the osteoclast is able to phagocytose material. Thus the necessity for a mononuclear cell to assist in phagocytosis and in degradation is unlikely.

Another possibility, is that PTH may act on a subpopulation of osseous cells to promote the production of factors which secondarily would alter osteoclast action. The supernatant of leukocyte cultures stimulated by antigen or by phytohemagglutinin (PHA) induced osteoclastic resorption of fetal rat bone in an organ culture system (Horton et al., '72). The substance referred to as osteoclast activating factor (OAF) is released by leukocytes (Luben et al., '74; Trummel et al., '75). Another possible candidate for the PTH-binding connective tissue mononuclear cell which may, with the classical preosteoclast described previously, be the same cell, or altogether two different cells. Lymphocyte enriched cellular populations obtained frommononuclear leukocytes bind radioiodinated bPTH (Perry et al., '84).

Role of competitive binding of ^{125}I-bPTH to osteoblast

By light microscopic observations, bone resorption which occurs during the growth process and secondary ossification has long been associated with osteoclastic action (Kolliker, 1873). Cellular specialization as seen at the electron microscope level, especially the ruffled border, has contributed to establishing firmly the active role of the osteoclast in the osteolytic process (Scott and Pease, '56; Holtrop and King, '77). However, PTH binds to osteoblasts in experiments in vitro and in vivo. Several possible functional consequences of this binding may be suggested, but the exact role of PTH binding to osteoblasts is uncertain.

For bone to be resorbed, there must be dissolution of the mineral and the bone matrix. It has been proposed that dissolution of mineral is the result of acid phosphatase, an enzyme released by the osteoclast (Vaes, '68), whereas the degradation of the matrix is the result of collagenase action (see review in Holtrop and King, '77). Collagenase is released from the culture medium after stimulation of bone resorption by PTE (Sakamoto et al., '75). A possible direct action of PTH on osteoblasts could be the modulation of collagenase metabolism or its release from the osteoblasts at the bone-cell interface, with the subsequent liberation of calcium ions into the bone fluid. However, the hormone could act intracellularly to degrade synthesized collagen prior to its secretion.

Although stimulation of bone formation by PTH has long been established (Selye, '32), more attention has recently been focussed on the anabolic action of PTH in view of the fact that osteoblasts , the bone forming cells, are targets for PTH, prostaglandins, and 1,25-Vit D_3

(see review in Rodan and Martin, '81). Cameron and co-workers have reported PTH induced changes in osteoblasts, including swollen mitochondria, and the presence of various dense bodies in the cytoplasm including lipid droplets (Cameron et al., '67). Others have reported a PTH-induced decrease in labeled proline incorporation into collagenase digestible proteins (Dietrich et al., '76).

In adult animals, there is little bone formation. In view of the position of the osteoblasts at the interface between the bone matrix fluid and the interstitial fluid compartment, PTH binding to osteoblasts could be involved in the rapid transfer of calcium from the bone fluid to the interstitial compartment.

Another possible role of PTH binding to osteoblasts could be the modulation of a whole array of osteogenic or osteolytic factors. Indeed, the hypercalcemic response could be, at least in part, secondary to the release of mineralization inhibitors or suppression of mineralization factors which could contribute to reduced calcium deposition in the skeleton. Some of these factors have been identified, eg. gamma-carboxyglutamic acid containing protein, also called osteocalcin (Price et al., '81) and phosphoproteins such as osteonectin (Lee and Glimcher, '81; Termine et al., '81). These newly discovered non-collagenous proteins which appear to interact with both collagen and the mineral phase may well have a great influence on the mineralization process.

In any case, the integrated control of calcium homeostasis of bone formation and of bone resorption certainly involves modulation of more

than one cellular process and probably affects more than one tissue. For the purpose of achieving calcium homeostasis PTH may act by inhibiting anabolic functions such as collagen synthesis, or alkaline phosphatase activity thereby reducing the drain of extracellular fluid calcium (Rodan and Martin, '81).

Calcitonin

Although *in vitro* studies suggest that calcitonin binds to the osteoblast layer of the periosteum and to mononuclear cells of the bone marrow (Rao et al., '81), efforts to demonstrate calcitonin's action on functions other than bone resorption have been unconvincing. The CT immunoreactivity detected over the osteoblast layer of the periosteum and over some mononuclear cells in bone marrow, has a certain degree of unspecificity, as all cells of bone seem to show the CT immumoreactive reaction. Moreover, at the light microscopic level, the degree of uncertainty associated with identifying mononuclear cells needs to be taken into consideration. In electron microscope investigation, mononuclear osteoclasts have been reported (Lucht, '72).

On the other hand, biochemical, histochemical, and ultrastructural studies all support the theory that in bone, calcitonin inhibits the osteoclastic-osteolytic function. In perfused cat bone, calcitonin causes a net retention of calcium (MacIntyre et al., '67) and decreases the efflux of calcium from bone even in the absence of PTH (Holtrop et al., '74). Morphological evidence suggests that the inhibition of bone resorption by CT is directly related to a rapid response at the ruffled border of osteoclasts. Twenty four hours after CT treatment, there is a flattening of the ruffled border and a loss of

the cytoplasmic coating (Kallio et al., '72; Lucht, '73).

In bone tissue, calcitonin promotes the production of cAMP (Marcus et al., '71). The calcitonin-dependent cAMP production is greater in preparations enriched in bone cells with adhering properties as obtained by sequential digestion of mouse calvaria, which suggests a particular action on osteoclasts (Wong and Cohn, '75). Competitive specific binding of CT to osteoclasts has been demonstrated by radioautographic (Warshawsky et al., '80), as well as by immunohistochemical methods (Rao et al., '81). The present study using the *in vivo* radioautographic method has verified that the same is true for mice.

Role of competitive binding of ^{125}I-sCT to osteoclasts

Competitive specific binding of ^{125}I-sCT has been localized over the cell surface of osteoclasts thus confirming the well established direct action of CT on this bone cell. It has been demonstrated that CT interferes not only with the synthesis, but with the release of lysosomal enzymes as well. Since osteoclasts from calcitonin treated bone are able to take up organic macromolecule, even though the ruffled border has disappeared, the particular effect of calcitonin appears to be a cessation of exocytosis with the concomitant disappearance of the ruffled border (Lucht and Norgaard, 77).

Binding to renal tissue

The kidney, in addition to removing the waste products of metabolism and potentially harmful substances from the blood, plays an important role in regulating fluid and the acid-base balance. Moreover, it contributes to the maintenance of electrolyte concentration in the body fluids. These functions are achieved by conserving some

substances and eliminating others. Among the conserved substances are water, sugars, amino acids, and electrolytes such as sodium, potassium, bicarbonate and chloride.

The functional unit of this organ is the tubular nephron which consists of several morphologically distinct segments each with a characteristic configuration and occupying a definite position in the cortex or the medulla. Moreover, each segment is lined with a particular type of epithelium.

Many of the kidney functions are regulated by hormones which modify the urinary composition and regulate the excretion rate of definite ions or solute species by acting on restricted portions of the functional unit so as to ensure body fluid homeostasis (Morel, '83).

The classic description of the physiological role of parathyroid hormone emphasizes the character of PTH as an agent of bone destruction (Thomson and Collip, '32). As much as 10^{-6} g/ml of PTH are required to significantly increase bone breakdown when the normal level of PTH ranges from 10^{-10} to 10^{-12} g/ml (Parsons and Potts, '72). Recently, particular interest has been attached to more sensitive responses which do not involve the skeleton. Moreover, because of the relatively late evolution of bone in fish, the primitive PTH-like factor would most likely have evolved in association with calcium regulation by the gills and kidneys (Parsons, '79), which could have retained some of this action in mammalian kidneys. The PTH generated action in the kidney may play an important role in the early component of the hypercalcemic response.

The role of the parathyroid hormone in the kidney

PTH stimulates calcium influx into kidney cells and activates adenylate cyclase (Borle, '68) which brings about an increase in renal tubular cAMP (Melson et al., '70). The distribution of the PTH-adenylate cyclase responsive system has been localized predominantly to the renal cortex (Chase and Aurbach, '68; Sutcliffe et al., '73; Canterbury et al., '73).

Development of a microassay for adenylate cyclase made possible the determination *in vitro* of the site of hormone action in a single well localized piece of the tubular nephron (Imbert et al., '75; see review in Morel, '83). By combining nephron microdissection with the adenyl cyclase microassay, it was shown that the synthetic fragment of bPTH stimulates adenyl cyclase activity in (1) the proximal tubule (PCT + PR), (2) in the cortical portion of the thick ascending limb (CAL), (3) in the granular portion of the distal convoluted tubule (DCT_g), and (4) in the granular portion of the cortical collecting tubule (CCT_g) of the rabbit nephron (Morel et al., '76). The pattern of adenyl cyclase activation in rats ressembles that found in rabbits (Morel, '81).

By the competitive *in vitro* approach, specific binding has been demonstrated in the kidney cortex (DiBella et al., '74). Using the *in vivo* competitive specific binding approach reported in this thesis, binding for ^{125}I-bPTH to presumably high affinity, low capacity sites, has been demonstrated in (1) the glomeruli, (2) on the anti-luminal aspect of the proximal, and (3) in certain segments of the distal tubular portion of the rat nephron. Non-competitive binding to presumably low affinity, low specificity, high capacity sites, has been

localized to structures located at the luminal aspect of the cells of the early portion of the proximal tubule.

The role of calcitonin in the kidney

Although it was shown that the hypocalcemic action of calcitonin is unaltered after nephrectomy (Munson et al., '66), the kidney plays a role in the generation of the hypocalcemic response in normal animals. In man (Bijvoet et al., '71) and in rats (Milhaud and Moukhtar, '66; Foster et al., '72) calcitonin induced calciuria. Although calcitonin appears to increase directly the urinary clearance of Ca^{++} some investigators have denied a CT effect on renal calcium excretion (Clark and Kenny, '69; Puschett et al., '74).

In kidney homogenates, CT promotes the production of cAMP (Marcus et al., '71). In rats, or mice, with the use of nephron microdissection and adenylate cyclase microassay, a high concentration of sCT induces low, but statistically significant adenylate cyclase activity in PCT, but not in the straight tubule (PR) of the rat (Kawashiwa et al., '81). In both rabbits and rats the enzyme system is activated in the medullary ascending limb (MAL), in the cortical thick ascending limb (CAL), and in the bright portion of the distal convoluted tubule (DCT_b) (Chabardes et al., '76; Ardaillou, '78).

Renal plasma membrane fractions prepared by homogenization and differential centrifugation in an isotonic sucrose solution contained adenylate cyclase activity positive for ^{125}I-sCT. The binding was inhibited by unlabeled sCT but not by ACTH or growth hormone (Marx et al., '72).

Using the in vivo competitive specific binding approach, binding to presumably high affinity, high specificity low capacity sites was found to be related to definite segments of the distal portion of the nephron in rats and mice. Non-competitive binding, of ^{125}I-sCT, as for ^{125}I-bPTH, to high capacity, presumably low affinity sites, was localized to the vesicular and vacuolar structures at the luminal aspect of the cells in the early portion of the proximal tubule.

Significance of the binding of ^{125}I-bPTH to the renal corpuscle

Competitives binding for ^{125}I-bPTH to podocytes. Competitive specific binding of ^{125}I-bPTH to low capacity, presumably high affinity, high specificity sites, was localized in electron microscope radioautographs to the primary foot processes of the podocytes of the renal corpuscles (glomeruli). It has been proposed that the glomerular podocytes have a potential for monitoring the filtration slit area available for solute passage across the glomerular wall. The filtration barrier consists of the fenestrated endothelium, the basal lamina, and the slit pores between the interdigitations of podocyte foot processes of the visceral epithelium. PTH could be involved in modifying the shape of the foot processes by expanding and contracting the base of these structures (Andrew, '81). Such an alteration in shape could regulate the thin membrane or slit diaphram which bridges the gap between adjacent foot processes (600 Å).

Binding for ^{125}I-bPTH to the mesangial cells.

Binding of ^{125}I-bPTH(1-84) to mesangial cells is not necessarily distributed to the cell surface and the binding to that cell is relatively low. This observation suggests caution with regard to identifying this reaction as associated with recognition sites. These cells are capable of internalizing compounds such as ferritin, colloidal gold, and immune complexes (Mauer et al., '72). A possible role in structural support to maintain the capillary loops in their proper anatomical relationship within the glomerular tuft, has been discussed. Because they contain contractile elements, they have been implicated in glomerular contraction. Their exact role in PTH metabolism and action remains to be established.

Binding of ^{125}I-bPTH to renal interstitial cells

^{125}I-bPTH(1-84) binds to cells in the kidney interstitium, called the reno-medullary interstitial cells. As many as three reno-medullary interstitial cells have been described on the basis of ultrastructural studies (Bohman, '80). Type 1 is the lipid containing interstitial cell. Type 2 is a lymphocyte-like cell, and type 3 is a pericyte (Bohman, '74). Type 1 cells are abundant in the inner stripe of the outer medulla and they are the only type in the inner zone of the medulla (Bohman, '80). The lipid-containing interstitial cell that binds bPTH may be involved in prostaglandin metabolism.

Role of competitive binding of bPTH and sCT to the tubular nephron

Proximal tubule. In the proximal tubule, PTH simultaneously reduces sodium, calcium, phosphate, and bicarbonate reabsorption. A major consequence is a substantial loss of phosphate, which is accompanied by an increase in bicarbonate excretion (Agus et al., '73). The phosphaturic effect of PTH might be the result of a primary inhibition of sodium reabsorption probably mediated by adenylate cyclase stimulation in the proximal tubular nephron (Agus et al., '71) and distal tubular nephron (Agus et al., '73) sites. This hypothesis is supported by the observation that the proximal tubule reaction has been associated with an inhibition of the sodium-phosphate co-transport process (Evers et al., '78). Another possible PTH-mediated process at this locus would be the 1-hydroxylation of 25-hydroxycholecalciferol (Brunette et al., '78) In patients with primary hypoparathyroidism, who have elevated level of PTH, there is an increase in the circulating level of 1, 25- Vit D_3 (Haussler et al., '75).

Distal tubule. In the distal tubular portion of the nephron, calcium absorption is markedly enhanced by PTH (see review in Parsons, 79). Depletion of endogenous PTH, in a hypoparathyroid subject is accompanied by a loss of a large quantity of calcium into the urine, even in the face of quite severe hypocalcemia.

The role of calcitonin in the distal tubule is unknown, however, since it is a hypocalcemic factor, and it may induce calciuria, it is likely that calcitonin acts on the distal tubule by inhibiting the PTH-induced calcium reabsorption by DCT-cells (the thick ascending limb is composed essentially of the DCT-cells). It may also play a role in

sodium transport. However, the binding of calcitonin to the three morphologically distinct cells in the distal tubule, the DCT-cell, the CNT-cell, and the intercalated cell, suggests more than one function for this hormone.

Non-competitive binding to the tubular nephron

The study of hormone binding in the proximal tubule portion of the nephron is complicated because, at this site, PTH occurs peritubularly as well as by uptake of glomerular filtrate (Martin et al., '77). The nature of the antiluminal reaction has been characterized in the in vivo system as competitive specific binding to presumably high affinity, low capacity receptor sites. The antiluminal reaction is most probably involved in at least part of the generation of the bio-response. The luminal reaction, localized to the early portion of the proximal region, is much stronger, and is associated with the membranous elements of an elaborate vacuolar system consisting of coated pits, coated vesicles, and vacuoles of various size. These may be empty or may contain fluffy material. The luminal reaction has been characterized as non-competitive binding to presumably low affinity, low specificity, high capacity binding sites. Similar binding and distribution for sCT (Fig. 71), PTH (Fig. 68), EGF (Polychronakos; unpublished data), insulin (Bergeron et al., '77), prolactin, and albumin (Maunsbach, '66a) have been reported. In all cases, the apparent protein specific reaction is with membrane located recognition sites. Iodide-125 intravenously injected did not result in reaction at this same locus.

The possible physiological role of PTH peritubular uptake has been discussed and is most probably an interaction which is bio-response related. The role of glomerular filtration uptake most likely involves protein catabolism. The highly developed endocytotic apparatus at the apical region of the cells in segment 1 of the proximal tubule, correlates well with avid absorption of filtered proteins.

Degradation of the hormone by cortical membrane, and most of the metabolic clearance of immunoreactive PTH, can be accounted for by the kidneys in normal dogs (Hruska et al., '75).

The kidney has been regarded as capable of producing the circulating fragments of bPTH(1-84) (Habener et al., '76). This is a remote possibility, as most of the endocytosed ligands are directed to the lysosomal system which suggests degradation.

The most likely reason for the high capacity luminal binding appears to be that of receptor mediated endocytosis which besides its obvious role in protein reabsorption and recycling, results in polypeptide hormone inactivation.

In general, caution must be used when comparing physiological data obtained in different species. The sites of action for a given hormone are not always located in the same nephron segment, as the anatomical organization of the nephron is different from one species to another. However, in both rats and mice, as well as in other species, the elaborate apical vacuolar system in the early proximal tubular segment suggests that the mechanism is generalized to all species.

In Vivo Competitive Binding Approach, Applications and Limitations

Binding site

Many of the properties expected from a true receptor interaction have been derived directly from pharmacological studies, and from the ever enlarging body of data which has accumulated as the result of studies of peptide hormone related binding. A major problem with the interpretation of the data resulting from such studies resides in the inability to differentiate between **binding to sites for the generation of the bio-response** from **binding to other sites not associated with biological functions.**

The *in vitro* approaches, used to characterize the ligand-cell interactions, have permitted the distinction of two extremes in binding interactions. One has been described as specific, high affinity, low capacity sites; the other as non-specific, low affinity, high capacity sites. Whereas specific binding is regarded as saturable; non-specific binding has, for the most, been regarded as an unsaturable component, at least with the use of *in vitro* studies (see review in Roth, '73; Kahn, '76).

The above distinctions must be considered with care. Competitive binding has been demonstrated on the test tubes and filters used in the *in vitro* experiments, and non-competitive binding may be so great as to mask the low capacity specific binding in the preparation. Homogenates and membrane preparation are made from whole tissue and contain a mixture of receptors for different hormones or even receptors for the same hormone but with variable affinity. The interpretation of the data is complicated by the source of the receptors. Heterogenous

populations of receptors have also been suggested by the atypical Scatchard plots, which indicate more than one class of receptor. It is likely that in receptor preparations, both those receptors for biological action, and those for inactivation, are present.

Attempts have been made to accurately describe the non-saturable components of the dose-response curves. In preparations in vitro, it has been equated with physical absorption to glass ware, or filters used in the experiments, and it is believed to be non-receptor related. In fact the situation is far more complicated, because the nature of the non-specific binding obviously differs according to the approach and system used.

One cannot neglect the fact that all animal cells have the ability to ingest extracellular materials (Palade, '56) by trapping them within inward foldings of the plasma membrane that pinch off from the surface to form intracellular vesicles, a phenomena referred to as endocytosis (Silverstein et al., '77). Fluid phase endocytosis, also known as pinocytosis, is a non-selective uptake of molecules. It has been differentiated from adsorptive endocytosis, a process whereby molecules are first selectively adsorbed at the cell surface sites prior to internalization. The process is now currently referred to as receptor mediated endocytosis, and has been described for a variety of substances including lipoproteins (LDL) (Goldstein et al., '79), asialoglygoproteins (Geuze et al., '82), and hormones (Posner et al., '82). Consequently, there is a need to differentiate between several types of receptors among which are those related to bioresponse generation, and those related to receptor mediated endocytosis.

No single property serves to identify receptor interactions. Several criteria , which have their origin in the various approaches, have been used to study the hormone-receptor interaction. When taken together they serve to distinguish receptor bio-response related from non-bio-response related interaction.

Optimally when studying ligand binding, it is desirable to examine (1) tissue distribution, and (2) binding characteristics including (saturability, reversibility, affinity, specificity, and capacity). Moreover, correlation of binding data with biological data expressed in dose-response curves, is essential for complete interpretation and understanding of receptor mediated biological phenomena, all of which involve an initial recognition event.

Activation of adenylate cyclase system

The characterization of a polypeptide hormone receptor as bio-response related, has been associated with the ability of the receptor preparation to activate the adenylate cyclase system (Sutherland et al., '68), a system in which the hormone uses cyclic AMP as a second messenger. Insulin, growth hormone, prolactin, and various growth factor-cell surface interactions do not lead to activation of the adenylate cyclase system (see review in Posner et al., '82), and therefore cannot be employed for assessment of the association of the receptor with a biological response.

Bio-response generation versus translocation process

Membrane receptors have been described as macromolecules with dual functions of (1) recognition and (2) cellular activation which brings about a well defined bio-response. Recognition sites participating in

the generation of the bio-response may be distinguished from others which may occur solely for selective cellular uptake. The former process has been referred to as receptor mediated endocytosis whereby proteins bind to receptors on cell surface and are rapidly internalized before they dissociate from their receptors. Biologically important molecules known to be taken up by this mechanism include plasma transport proteins such as low density lipoproteins (LDL) (Goldstein et al., '79), and transcobalamin II (Youngdhal-Turner et al., '78). Following cell surface interaction, the LDL-complex is translocated into the cell. Although the interaction is not considered a bio-response related process, strict chemical high specificity and high affinity properties of the specific ligand-receptor interaction have been conferred to this interaction. Hence many receptors for various systems, including the LDL and the prostaglandins, which involve an interaction with binding sites on the cell surface, appear in many respects similar to the polypeptide hormone receptor interaction.

Consequently, characteristics of the recognition bio-response related binding, and those of the translocation, overlap in terms of capacity, affinity, and reversibility.

In terms of structures in which they are translocated in the cell, the low capacity, high affinity, high specificity LDL binding to fibroblasts overlaps with the high capacity, presumably low affinity, low specificity of polypeptide hormone binding to the vacuolar system at the apex of the proximal tubular portion of the nephron. Similarly, the LDL system and the proximal tubular binding are concentrated in coated pits and form intracellular coated vesicles prior to the translocation process.

Four properties collectively define receptor mediated endocytosis (Goldstein et al., '79). (1) The binding component of the cell surface is a receptor in the strict sense that it is a molecule whose function is to bind an endogenous ligand and thereby achieve a physiological effect. (2) Internalization of the ligand is effectively coupled to binding. Once the protein is bound to the receptor, the half-life for internalization is less than 10 minutes. (3) In all cases for which ultrastructural data is available, the receptor bound protein enters the cell through coated pits. (4) Finally, the internalized proteins are usually delivered to the lysosomes where they are degraded to amino acids, although occasionally processing of the internalized protein involves delivery to cellular structures other than lysosomes.

In the broadest sense, the non-competitive binding related to the endocytotic system of the proximal tubular cells (which most probably is for protein translocation purposes) can be regarded as a receptor interaction, at least in terms of a recognition site.

Classes of receptors

Radiolabeled polypeptide hormone binding has been studied with the use of a direct in vivo approach two minutes after intravenous injection. When coupled to light and electron microscope radioautography, the technique permits maximum resolution in terms of topographical localization of the labelled ligand. The reaction can be assigned to the morphologically different cells that constitute a tissue, or can be analyzed simultaneously in various tissues obtained from the same animal. The relative ability of related unlabeled hormone

to compete with the labeled hormone for receptor occupancy over the different cell types in one tissue can be determined. The observation can be extended to various tissues and correlations can be established in terms of binding distribution, internalization, and the competitive ability of unlabeled ligand for receptor occupancy.

In general, within two minutes after injection, the binding of the labeled hormone is visualized on the cell surface, or on the membranous aspect of the vacuolar elements in agreement with the premise that the initial site of polypeptide hormone interaction is located at the cell surface. In a given tissue, intravenously injected iodinated hormone binds to more than one cell type. Based on qualitative observations at the electron microscope level, and on the results of a first estimation of the silver grains, the binding of radiolabeled hormone to different cell types may be maximally (95%), partially (50%), or minimally (10%, or less), inhibited by co-injection of excess related unlabeled hormone.

In the current literature, two classes of receptors have been described on the basis of changes in cell behavior or on the basis of metabolic activity following the recognition interaction. Class I receptors lead to change in cellular behavior; although internalization occurs, it appears not to be a pre-requisite for ligand function. The major role of class II receptors is to mediate ligand internalization. The binding does not, per se, lead to alteration of the cell activity. Modification of the cell metabolism, if it occurs at all, is the consequence of ligand metabolism (Kaplan, '81).

In this classification, Class II receptors, according to Kaplan, include the traditional hormone receptor and receptors for other humoral non-hormonal ligands. The interactions between class I

receptors and ligands are conventionally treated as reversible reactions subject to up- and down- regulation phenomena, and are not reutilizable receptors. Class II receptors have been referred to as scavenger receptors. They do not exhibit any measurable rate of dissociation and appear to be reutilizable receptors. The Class II ligand-receptor interactions have been localized to coated pits and do not lead to any alteration in cell behavior. Human fibroblasts incubated in vitro exhibit identical rates of macromolecular synthesis and growth in the presence and in the absence of low density lipoproteins (Goldstein and Brown, '74), even though such lipoproteins can interact with Class II receptors on these cells. Cells incubated in the presence or absence of transcobalamin II, which also interacts with Class II receptors, also exhibit similar growth rates (Mahoney et al., '71).

If the receptors were to be classified on the basis of the various events which follow the ligand-recognition step, then several classes of receptors may exist or co-exist on the same cell surface.

Ligand interactions of the recognition-action type, as well as recognition-translocation types, probably both involve a recognition event with elements situated at the cell surface, at least for non-lipid non-hydrophobic elements. The biological events that occur beyond the recognition process certainly involves overlapping mechanisms and probably heterogeneity in those mechanisms by which they achieved the bio-responses. No matter what the class of receptor, binding to any recognition site is likely to bring about numerous membrane changes which at one extreme may result in a single event, and at the other extreme, multiple cellular events.

In the case of recognition-action binding, the formation of the cell surface complex may generate various changes at the cell membrane; additionally the hormone-receptor complex may be internalized for subsequent action, or for simple inactivation, or for receptor recycling purposes. Among the changes seen at the cell membrane is an apparent modulation in intracellular transport of calcium (Chausmer et al., '72) Additionally, ligand binding is followed by ligand internalization (Steinman et al., '83), and this appears to be occuring at all times as in the present experiments, where at two minutes, there was always a substantial amount of ligand internalization. What occurs past the internalization process is likely to be different according to the ultimate purpose of the internalization.

The same subcellular endocytotic structure may serve for the entrance of different components. The contents of the membrane components of the endocytotic vesicles may be sorted out from one another. Endocytosed content may accumulate in the cell while the container can move into or out of the cell after one or more fusion events with other endocytotic vacuoles, lysosomes or the Golgi apparatus. The ligand may be rapidly degraded as the result of lysosomal action, or the protein may be directed towards other specific subcellular organelles.

An interesting possibility, as far as application of the <u>in vivo</u> competitive binding approach is concerned, is that by using this approach, receptors may be classified on the basis of the relative ability of unlabeled hormone to compete for receptor occupancy. Hence, three classes of receptors may be identified, corresponding presumably to low, intermediate, and high capacity sites, as the binding of the

labeled ligand may be maximally, partially or apparently not inhibited, at least at the concentration of excess unlabeled hormone co-injected with the labeled hormone in the **Control** animals.

In the present approach, it is the recognition function of the receptor which was the focus of our attention. That is, the interaction with the recognition subunit of the macromolecular receptor, the cell surface related phenomena, or the earliest event in the hormone-cell interaction.

Nevertheless, electron microscope observations indicated that two minutes after intravenous injection, in all cases, a certain amount of the labelled hormone had been internalized. Two classes of receptor-interaction could be described based on the structure into which the hormone was subsequently internalized. In one case it was associated with the cell surface and small vesicles found beneath the cell surface, and dense bodies (Figs. 14, 15, 26-28, 35-40, 64, 65, 73, 79). In the other case, it was cell surface related, related to coated pits, or associated with the membranous elements of variable size vacuoles, sometime containing fluffy material, or to the membranous aspect of dense bodies identified as lysosomes (Figs. 16-18, 30-32, 68, 71, 77). This was mostly seen over sinusoidal endothelial cells and at the luminal aspect of the proximal tubule cells.

Although certainly not sufficient for a complete description of the ligand-cell surface interactions, the addition of an <u>in vivo</u> morphological component to the physico-chemical biological approach may help elucidate the receptor mediated cellular processes, including the polypeptide hormone-receptor interaction.

Binding of CT in Osteopetrotic Rodents: Functional Considerations

Binding of ^{125}I-sCT to osteoclasts

Osteopetrosis is a disorder of the skeleton characterized by abnormal bone density and brittleness. In general, the marrow spaces are obliterated by osseo-cartilagenous trabeculae (Jaffe, '72). The pathogenesis of the disease remains uncertain, and it is still uncertain if there is over production of bone tissue or reduced resorption, and if the abnormal structure of the bone is due to defective osteoclasts, or to changes in the structure and composition of the calcified matrix (Bonucci et al., '75). The symmetrical sclerosis certainly indicates some form of disturbance in the mineralization process.

Barnicot (1945) was the first to investigate a possible endocrine basis for osteopetrosis. He observed that grey lethal mice tolerated doses of PTE that would have killed normal littermates (Barnicot, '45). Subsequent studies to identify a possible endocrine disorder have focussed primarily on the two major hormones involved in calcium homeostasis, parathyroid hormone and calcitonin. In osteopetrotic mutants, low plasma calcium, increased bone mass, and an increase in the number of parafollicular cells, suggests an hormonal excess of circulating calcitonin as a likely cause of osteopetrosis. The excess calcitonin hypothesis was abandoned, as most attempts to cure or to produce the condition on this endocrinologic basis have failed. Thus, removal of the source of calcitonin by neonatal thyroidectomy supplemented with thyroxine, did not reverse osteopetrosis in mice (see review in Marks and Walker, '76). Additionally, a high calcium diet fed to normal rats induced parafollicular hyperplasia, but it

did not induce osteopetrosis (Walker, '71). In one case, osteopetrosis was produced in TPTX rats by administration of thyrocalcitonin (Foster et al., '66), but in general, administration of calcitonin to intact or thyroidectomized mice or rats produced neither osteopetrosis nor an elevation in bone formation.

Attempts to increase PTH level by injection of PTE, or transplantation of parathyroid glands, have not cured the disease in mice or rats. Moreover, chronic treatment of mouse or rat mutants with PTE increases rather than decreases the density of the skeleton (Barnicot, '45; see review in Marks and Walker, '76). At the present time, it is believed that osteopetrosis is not the result of a primary endocrine or humoral defect.

In osteopetrotic mutants, significant alterations in osteoclast number and size, as well as in enzymatic activity have been reported, suggesting that the disorder could involve a target cell defect. The disease is cured in many cases when a source of competent osteoclasts is provided either temporarily by parabiosis or by marrow and spleen transplants (see review in Marks and Walker, '76).

The potential events that could alter the resorptive ability of the osteoclasts are multiple. Any abnormal event occuring in the life cycle of an osteoclast could lead to a reduction in cellular function. Among these are (1) the proliferation of the stem cell population, (2) the entry of the progeny into the vasculature, (3) the immigration to skeletal site for eventual differentiation, (4) the selection of the appropriate skeletal site for eventual differentiation, (5) the migration from the blood vessels to this site, (6) the differentiation and fusion of the precursors, and (7) the cellular activation, and

regulation by humoral agents such as the OAF, or hormones. Moreover, alteration in any of the multiple post-receptor events could also result in the inhibition of the expected bio-response (see review in Marks, '84).

Evidence of abnormal resorption involving bone modeling and remodeling, directed attention towards the assessment of osteoclastic function and regulation. The in vivo competitive-specific binding approach using radioautography showed low capacity, presumably high affinity specific binding sites for ^{125}I-sCT on the osteoclast cell surface by the radioautographic approach (Warshawsky et al., '80). In the present study, an attempt was made to detect alteration in ^{125}I-sCT binding to osteoclasts in 4 different osteopetrotic mutant models. Morphological as well as quantitative observations were compared to those obtained in the osseous tissue of normal siblings injected with an equal amount of radiolabeled ^{125}I-sCT.

Whereas in the oc/oc, and in the ia/ia mutant rodents, there was a significant reduction in ^{125}I-sCT binding, in the op/op and in the mi/mi mice, there was a significant increase in ^{125}I-sCT binding. It is tempting, when considering only the quantitative results, to discuss the alteration in binding as two major groups of alterations. The available scientific literature dealing with these mutants, as well as ultrastructural observations, indicate a further heterogeneity in each of these categories. However, in the osteopetrotic op/op mice, it was found that the increase in binding is paralleled by an increase in cell surface. In this mutation, the osteoclast cell surface is arranged in outward projections which resemble bulb-like structures, similar in mor-

phology to pseudopods of the giant-cell tumor of bone (Hanaoka et al., '70). In contrast, the cell surface in the microphthalmic mi/mi mutant appears normal.

Morphologically, the osteoclasts in the osteosclerotic oc/oc, and incisor absent ia/ia rodents are similar. Heterogeneity in these is suggested by the observation that in the os/os mutation most animals die at an early age, and the condition cannot be cured by cellular transplants (Marks and Seifert ;cited in Marks, '84). In ia/ia mutants, there is spontaneous recovery from the resorptive failure, and bone resorption can be restored after parabiotic union of ia/ia rats with normal littermates (Marks and Schneider, 82). Thus, it is preferable to discuss the alteration in hormone-receptor interactions separately in each individual mutant, as there is strong evidence that osteopetrosis in these four types is the result of different sets of events.

Osteopetrotic op/op mice

Quantitative data indicates an increase in calcitonin binding over osteoclasts in this mutation. Increase in binding is readily associated with an up-regulation phenomena, that is, a presumed increase in receptor number which could be the result of extracellular or intracellular regulating factors. If the increase in the number of receptors was the result of homospecific regulation, one would expect to find a decrease in the circulating level of calcitonin. This is an unlikely possibility, as in this mutation there is a six fold increase in the parafollicular cells and the plasma level of calcitonin was normal (Marks, '76; unpublished data cited in Marks, '84).

Because there is remission at 90 days (Lane, '75), and since the con-

dition cannot be cured by bone marrow or spleen transplants, it appears that the defect does not lie in the competence of the progenitor population (Marks et al., '84). Moreover, at 90 days there is remission of the condition indicating that in this model the osteoclast itself is probably normal. A possible abnormality in the physico-chemical environment must therefore be considered. In the osteopetrotic op/op mutant mice, the resorptive function of the osteoclasts appears diminished. There is a decrease in the number of cells, as well as a decrease in activity (Marks and Lane, '76). This decrease in function is accompanied by an increase in the ^{125}I-sCT binding as shown in this thesis.

The presumed increase in cell surface receptors is associated with an increase in cell surface. Consequently, if the concentration were to be expressed per cell surface area, there might not be any difference in ^{125}I-sCT binding per cell surface area when binding in the op/op mutant is compared to binding in the normal littermate. Nevertheless, increased numbers of binding sites per cell could render the osteoclast more susceptible to a **normal** circulating level of calcitonin. This model could therefore represent a situation in which reduced activity of the osteoclast is, at least in part, related to increased efficacy of circulating CT.

The increase in cell surface complexity may be compared to the increase in cell surface complexity reported with scanning electron microscopy on osteoclasts in short term culture. These show additional free surface ruffles which are not encountered in specimens taken directly from living animals (Jones and Boyde, '77). This may be additional evidence for the hypothesis of an altered physico-chemical environment.

Microphthalmic mi/mi mice

In this mutant the apparent reduction in resorption is not the result of an abnormality in the environment in which the osteoclast lives, as the condition is cured after establishment of cross circulation with a normal littermate (Walker, '72) as well as by bone marrow and spleen transplants (Walker, '75a). As in the case of the op/op mice, an increase in cell surface receptors for ^{125}I-sCT has been found. Topographical distribution of the receptors indicates an increase in the receptor number per cell surface area. A three fold decrease in CT-induced cAMP production has, however, been reported (Kent et al., '79). Consequently, both the increase in receptors for sCT and an associated decrease in the transduction of the hormone-receptor signal to the catalytic unit may reflect, rather than cause, a fundamental abnormality in the osteoclast. Therefore, the efficacy of the circulating CT may not be enhanced in this model despite the increase in osteoclast receptors for the hormone.

Osteosclerotic oc/oc mice

In this mutant, the condition cannot be cured by bone marrow and spleen transplants (Marks and Seifert cited in Marks, '84). Since the osteoclasts are not deficient, but are present in excess numbers (Marks C.R.; cited in Marks, '84), reduction in resorption is not due to absence of progenitors, nor to the fusion of precursors, nor to migration to the site of resorption.

The absence of ^{125}I-sCT binding to these cells suggests a severe down-regulation of the CT receptors. This alteration could be through homospecific regulation, as a six fold increase in the number of

parafolicular cells has been reported, although the levels of circulating calcitonin has not been reported. Alternately, the reduction in CT receptors may reflect widespread abnormalities of the osteoclasts in this mutant induced by other environmental events, as osteopetrosis in this mutant cannot be cured by cell transfer.

Incisor absent ia/ia rats

With the incisor absent ia/ia mutation there is a spontaneous recovery from the resorptive failure. In this mutation, the osteoclast is synthesizing acid phosphatase, but the lytic enzyme is not released at the bone-cell interface. At the time of remission, the acid phosphatase is released but the ruffled border is not formed (Marks, '73). Because resorption can be restored following parabiotic union of the ia/ia rats with a normal littermate, as well as by bone marrow and spleen transplants (Marks, '76; Marks and Schneider, '82), the primary defect is apparently not in the physico-chemical environment but in the target cells themselves.

Absence in binding to osteoclasts in these mutants suggests absent or defective receptors for calcitonin. In this case the binding defect most probably reflects a primary abnormality in the osteoclast. This is a likely possibility as upon remission, there is an increase of 20% in resorption in the ia/ia mutant with an increase in acid phosphatase and in cell size and number. The possibility of homospecific down-regulation is unlikely in this mutant, because in contrast to oc/oc mice, the number of parafollicular cells has been reported to be normal.

Liver and kidney functions in osteopetrotic rodents

Liver

In normal animals, sCT binds to liver and kidney tissues. Thus it was important to determine whether the altered ^{125}I-sCT binding to osseous tissue was accompanied by abnormal binding in other target tissues. Comparative CT binding studies indicate a significant increase in sCT binding in the liver of osteopetrotic op/op, and microphthalmic mi/mi mice which parallels the increase in binding in the osseous tissue of these mutants. The physiological significance of the increase in binding is unclear.

Rosen and Haymovits (1972) examined the liver in an osteopetrotic human subject. They attempted to assess the activity of enzymes in the condition based on the hypothesis that any inborn lysosomal disease is believed to extend to all cell types in which the missing, deficient, or inactive enzyme is controlled by the same gene. They did not report any enzyme abnormality but documented an increase in granules in liver mitochondria, presumably indicative of an increased concentration of calcium-phosphate salt, and an increase in acid-beta-glycophosphatase (Rosen and Haymovits, '72). Their first hypothesis may have had some truth to it. Contrary to their report, our observations of the liver structures at the electron microscope level have shown that in the op/op mice cytoplasmic bodies, presumably secondary lysosomes, are abnormally electron dense and numerous in all cells of the hepatic tissue including hepatocytes (Fig. 19) sinusoidal and Kupffer cells (Figs. 19, 20).

Kidney

In all of the osteopetrotic cases examined in this study, based on

morphological observations, there is some form of kidney abnormality. In segment 1 of the proximal portion of the nephron in the op/op mutant there is a decrease in the ability to remove labeled proteins from the glomerular filtrate (Graph 29), whereas in the mi/mi mutants, there is an increase in protein resorption by the endocytotic system at the apice of the proximal tubular cells (Graph 29). In the oc/oc and in the ia/ia mutant rodents, the significant reduction in ^{125}I-sCT binding in bone is accompanied by a large amount of structural abnormalities in both the proximal and the distal tubular segments (Figs. 90-101).

The significance of these observations are unknown at the present time. However, many cases of osteopetrosis, especially in human, have been already associated with renal tubular acidosis (Guibaud et al., '72; Vainsel et al., '72; Whyte et al., '80). It is still not clear what part electrolyte physiology plays in osteoid mineralization or what is the involvement of renal function in that process, but is likely, that the absence of calcitonin binding in the distal portion of the nephron, the abnormal protein handling by the proximal tubular portion of the nephron, and the great many structural alterations in the kidney of these rodents results in multiple functional abnormalities which if not directly involved in the pathogenesis of the condition may contribute to aggrevate its expression.

Therefore the role that the abnormalities in structures and in calcitonin binding play in the osteopetrosis is unknown at the present time. However, the above observations allows for a certain amount of speculations.

In osteopetrotic and in microphthalmic mice, there is an increase in ^{125}I-sCT binding to the osteoclast cell surface. Although this

increase is paralleled by an increase in binding in the liver tissue, this is not the case for renal tissue. In the osteosclerotic oc/oc mice and in the incisor absent ia/ia rats, absence of ^{125}I-sCT binding has been associated with a defective or a greatly reduced affinity for calcitonin in these mutants. The receptor abnormality is paralleled by identical renal receptor deficiency. This observation suggests that the defect may be genetic, that is, in the coding of the receptor molecule, or the architectural arrangement, or in the receptor insertion in the membrane of the cell.

An other interesting possibility resides in the fact that the structure and function of the receptors, like those of all other proteins, are heavily influenced by the physico-chemical environment. Disorder in lipid metebolism can affect the lipid matrix of the plasma membrane together with the structure and function of the proteins embedded in it. Abnormal lipid-containing structures have been noticed at least in the osseous and in the kidney cells of the op/op mutant mice (Fig. 54) (Marks, '82). The type and proportion of lipid component in the membrane strongly influences the function of the receptor and other integral proteins such as adenylate cyclase. The lipids of the membrane are rapidly exchangeable with those of the environment so that change in the plasma lipids could result in changes in the membrane lipids which in turn could affect the behavior of the membrane proteins. Moreover, lipids have been associated with calcification (see review in Wuthier, '82).

CONTRIBUTIONS

The work performed in requirement for the degree of Doctor of Philosophy has yielded the following contributions to knowledge;

Competitive binding for ^{125}I-sCT and ^{125}I-bPTH, to low capacity, presumably high affinity, high specificity recognition sites has been localized to individual cells in light and electron microscope radioautographs in liver, osseous and renal tissues in rodents.

Total binding, including non-competitive binding to high capacity presumably low affinity, low specificity recognition sites, has been described and illustrated in light and electron microscope radioautographs.

In extension of the studies done in normal rodents, the same approach was used to study CT binding in osteopetrotic rodents. The results of this study indicated a significant increase in CT binding to osteoclasts in osteopetrotic op/op and microphthalmic mi/mi mice. Radioautographs obtained from osteosclerotic oc/oc and incisor absent ia/ia rodents showed absence of CT binding over the osteoclasts of these two mutants.

Together with the alteration in CT binding, structural abnormalities in the renal tissue of these mutants have been illustrated.

A useful scheme for segmentation of the kidney tubule has been presented for the purpose of determining exactly which segments of the nephron contained hormone receptors for ^{125}I-sCT and ^{125}I-bPTH.

Observations using the *in vivo* radioautographic approach to study the hormone-receptor interaction suggest that this approach is usefull in differentiating between at least three classes of receptors.

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