DIRECT *IN VIVO* DEMONSTRATION BY RADIOAUTOGRAPHY OF SPECIFIC BINDING SITES FOR CALCITONIN IN SKELETAL AND RENAL TISSUES OF THE RAT

BY

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A thesis submitted to the Faculty of graduate studies and research in partial fulfillment of the requirements for the Degree of Master of Science.

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To my father because he always cared

Short title

IN VIVO SPECIFIC BINDING FOR CALCITONIN IN THE RAT

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ABSTRACT

Radioautography was employed to visualize the *in vivo* binding of calcitonin to receptors in rat tissues. Two minutes following intravenous injection of biologically active ^{125}I-salmon calcitonin, free hormone was separated from bound hormone by intracardiac perfusion with lactated Ringer's followed by fixation with 2.5% glutaraldehyde. Various tissues were removed and processed for light and electron microscope radioautography. These were compared to tissues removed from animals which received identical amounts of labeled hormone with a large excess of unlabeled calcitonin. Among the tissues investigated, kidney, bone and brain demonstrated labeling.

In kidney, most silver grains were located over vesicles below the brush border of cells of the proximal convoluted tubules. These grains were still present after simultaneous injection of excess unlabeled hormone and most likely represented low affinity nonspecific binding to sites involved with ingestion and degradation of hormone from the urinary filtrate. In contrast, grains localized to the basal surfaces of the ascending thick limb of the loop of Henle and the distal convoluted tubule cells were significantly reduced in number in control animals and represented high affinity, specific binding sites for the hormone. In bone, specific binding sites were found all over the cytoplasmic membrane of the osteoclasts but not on the ruffled border. These labeled cells were located at resorption sited examined in calvaria, in alveolar bone, and at the metaphyseal surfaces of the epiphyseal plate of the humerus and tibia. Specific binding sites for the hormone were also found in the subfornical organ of the brain; a site of binding not previously reported in the literature. This demonstration of the localization of ^{125}I-calcitonin *in situ* provides a new and sensitive approach for studying the interaction of calcium regulating hormones with their target cells.

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TITLE OF THE THESIS — Direct *In Vivo* Demonstration by Radioautography of Specific Binding Sites for Calcitonin in Skeletal and Renal Tissues of the Rat.

DEPARTMENT — Anatomy

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TIRE A PART

Nous avons utilisé la logique de l'essai pour sites de liaison de haute spécificité et les techniques radioautographiques afin de visualiser les récepteurs de calcitonine dans les tissus du rat. Deux minutes après l'injection intraveineuse de l'hormone marquée à l'iode 125, l'hormone non-liée a été séparée de l'hormone liée aux tissus par une perfusion intracardiaque amorcée avec une solution de Ringer suivie par la solution fixative. Les tissus d'intérêt ont été disséqués et préparés pour la radioautographie au microscope optique et électronique. Les radioautographies obtenues de ces tissus ont été comparées à celles de l'animal de contrôle auquel on avait injecté une quantité identique d'hormone marquée, mélangée à un excès d'hormone non-marquée, de telle façon que dans cet animal il y ait compétition entre l'hormone marquée et l'hormone non-marquée pour la liaison des récepteurs de haute affinité.

Dans le rein, la plupart des grains radioautographiques sont situés au-dessus des vacuoles juste sous la bordure en brosse des cellules des tubes contournés proximaux. Puisque ces grains n'ont pas été réduits en nombre par l'injection simultanée d'un excès de l'hormone non-marquée dans l'animal de contrôle, ils représentent probablement des sites de liaison de basse affinité d'ingestion et de dégradation de l'hormone provenant du filtrat glomérulaire. Au contraire, les grains que l'on a observés sur la surface basale des tubes contournés distaux ont été réduits en nombre de manière significative dans l'animal de contrôle et représentent donc des sites de liaison de haute affinité. Dans les tissus osseux, les sites de liaison de haute affinité et spécificité ont été localisés sur la membrane cytoplasmique de l'ostéoclaste. Les grains radioautographiques ne se trouvent pas associés à la membrane cytoplasmique de l'ostéoclaste formant la bordure striée. Finalement, des sites de haute affinité ont été démontrés dans l'organe subfornical du cerveau.

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INTRODUCTION

INTRODUCTION

The body, through its endocrine system, communicates information between cells. The endocrine cells release their messages into the blood stream, which delivers them indiscriminately to target and non-target tissues. The target cells contain specialized molecules, called receptors, that bind the hormone and subsequently mediate its cellular action. The receptors vary in their cellular localization, in the post-binding transfer of information, and in the particular hormone they recognize. There are three types of hormone receptors: 1. for polypeptide hormones, catecholamines, and releasing factors, the specific receptors are on the external surface of the plasma membrane of the target cells, 2. for steroid hormones the receptors are found initially in the soluble intracellular compartment of the cells, 3. for thyroid hormones the intracellular binding sites are found in the chromatin of the target cells.

POLYPEPTIDE HORMONE RECEPTOR

The type of receptor of concern in this investigation is the receptor for polypeptide hormones. As previously mentioned, these receptors are on the cell membrane (Cuatrecasas and Hollenberg, 1976), and the binding site is located on the outside aspect of the cell membrane. The receptors have also been found in a number of subcellular fractions such as Golgi fraction (Bergeron et al., 1978), and nucleus (Goldfine et al., 1976). They are predominantly proteins and are intimately associated with membrane lipids. They may also contain carbohydrates.

It has been demonstrated that, in certain cases, the interaction of peptidide hormone with the receptor results in activation of adenylate cyclase, a membrane bound enzyme which stimulates the conversion of ATP to adenosine - 3',5' - monophosphate. The latter serves as a second messenger in a sequence of events, still to be clarified, which ultimately culminates in the appropriate cellular response. In that sequence of events, the role of the receptor is to distinguish a signal given by a particular compound from a variety of circulating molecules and to relay this information in such a way that the appropriate cellular response follows. To recognize the signal, the receptor must show specificity, have an affinity for the hormone and an appropriately lower affinity for potentially similar and misleading signals.

RATIONALE OF THE *IN VIVO* SPECIFIC BINDING DETERMINATION

Affinity is a function of non-covalent binding which involves electrostatic interactions including hydrogen binding, hydrophobic interactions and Van der Waals forces. A simple way of considering affinity is to take a familiar system from basic chemistry, namely, the formation of a product from two reactants. As a consequence, the binding of hormone(H) with receptor(R) can be simply represented as $H + R \overset{k1}{\underset{}{\rightleftharpoons}} HR$. The rate at which this reaction proceeds is a function of the concentration of hormone [H] and receptor [R], and therefore, the inherent rate at which the hormone combines with its receptor is mathematically equal to $k1[H][R]$ in which case k1 is the rate constant. The hormone binding is usually reversible; this can be represented as $HR \overset{k2}{\rightleftharpoons} H + R$ and the rate at which this reaction proceeds is mathematically equal to $k2[HR]$. At equilibrium, the rates are equal and

therefore $k_2[HR]$ is equal to $k_1[H][R]$. The equilibrium dissociation constant (Kd), k_2/k_1 is equal to $[H][R] / [H]$. The reciprocal is the equilibrium association constant (Ka), k_1/k_2 and it is equal to $[HR]/[H][R]$. The Ka is also commonly referred to as the affinity constant, and the greater this value, the higher the affinity. Another way to express the affinity is using the relation Kd equals $[H][R]/[HR]$. When half of the receptors are occupied by the hormone, the Kd is then equal to $[H]$ and in this case the affinity is described as the free concentration of hormone necessary to half saturate the receptors of a given receptor preparation (Posner, 1975). Therefore, the lower the Kd value, the higher is the affinity.

The development of methods for separation and characterization of cellular components, the preparation of highly purified polypeptide hormone, and the availability of a simple procedure for iodination of hormone with high specific activity, enabled the direct study of polypeptide hormone-receptor interactions. Above all, the technical innovation developed in 1960 by Berson and Yallow in the radioimmunoassay field had a powerful impact on the direct study of these interactions. Although they never worked directly on receptors, investigators now working with peptide hormone receptors have directly applied the methods and technical approaches devised by them for radioimmunoassay (Yallow, 1978).

The studies indirectly associated with the present work involve the *in vitro* radioreceptor assay developed by Roth (1973). In this assay, a similar amount of membrane preparation, ideally containing an equal concentration of receptors, is added to a series of tubes, together with an appropriate amount of labeled hormone capable of binding all of the receptors present in the preparation. An excess of the unlabeled hormone which

competes for the limited number of receptor sites is added to half of these tubes establishing the affinity of the hormone for the receptor. In another series of tubes containing the same membrane preparation and the same labeled hormone, structurally dissimilar cold hormones are added. Even when present in great excess, these unrelated hormones cannot compete with the labeled species, thus establishing the specificity of the binding. After incubation, unbound hormone is separated from bound hormone by centrifugation or filtration of the preparation on a millipore filter. Quantitation of labeled hormone bound to receptor is achieved with a liquid scintillation counter and expressed in disintegrations per minute (dpm). In this assay, total binding is defined as the radioactivity retained in the absence of excess cold hormone, and nonspecific binding as that radioactivity retained in the presence of excess unlabeled hormone. Specific binding is ultimately obtained by subtraction of nonspecific binding from total binding. The rationale of the *in vitro* receptor assay is the foundation for the *in vivo* specific binding determination acheived in the present experiments. However, instead of using two series of test tubes, containing equal amounts of receptors, two animals of similar weight, sex and strain are used. Ideally, these rats contain the same number of receptors. The first animal, referred to as the experimental, is injected intravenously with an appropriate amount of the labeled hormone; the second animal, referred to as the control, is injected with the same amount of radioactive material mixed with an excess of the cold hormone which competes with the labeled species for the limited number of receptor sites. The specificity of the binding can also be demonstrated in the *in vivo* system, where the binding of labeled hormone will be competitively inhibited only by unlabeled

biologically related hormone and not by unrelated molecules. In the *in vivo* localization study, unbound hormone is separated by an appropriate technique involving the removal of circulating material by intracardiac perfusion initiated with a solution of lactated Ringer's. Histology and radioautography are used to determine the localization of the labeled material. Quantitation of the labeled material is expressed as the number of radioautographic silver grains counted over histological sections of equal thickness in equal areas delimited by an ocular grid with a light microscope. In the *in vivo* specific binding determination, the total binding is defined as the grain count over histological sections of the tissue of the experimental animal and the nonspecific binding is the count over histological sections of the control animal. Quantitatively, the specific binding is ascribed to structures which show a significant degree of competition, as evidenced by a significantly higher experimental value than the control. Qualitatively, specific binding occurs when silver grains are present over tissue of the experimental animal but not over identical sections and structures of the control animal.

With the *in vitro* system the nonspecific binding reflects either physical adherence of labeled hormone, or absorption to filters or test tubes employed in the assay. In the *in vivo* system, it reflects physiological adherance of the labeled hormone at its degradation site and adsorption to cells and intercellular spaces anywhere in the body where the circulating labeled hormone has access.

CALCITONIN

Historical Survey

In 1935, McLean and Hasting observed that, in a normal mammalian organism, the constancy of the plasma calcium concentration was maintained in spite of wide fluctuation in the intake and excretion of this ion, and they reported that the level of calcium was one of nature's physiological constants. Subsequently, it was shown that in the absence of the parathyroid glands the calcium level fell significantly and, as a consequence, major attention was directed towards defining the relationship between this gland and the plasma calcium concentration (Rasmussen and Pechet, 1970). In 1960, Sanderson, Marshall and Wilson observed that, in thyroparathyroidectomized dogs, there was an inability to handle either low or high levels of calcium. The results obtained by Copp and his colleagues in 1961, after they perfused the parathyroid and the thyroid glands together with blood high in calcium, lead them to propose that a second calcium regulating hormone existed and that it was found in the parathyroid. In 1963, and 1964, Hirsh and his colleagues demonstrated the presence of a hypocalcemic factor in the thyroid gland and subsequently named it thyrocalcitonin to distinguish it from calcitonin of possible parathyroid origin. One year latter, Foster (1964) demonstrated the presence of calcitonin secreting cells in the thyroid glands. Perhaps the most conclusive experiment was made by Care in 1965, where perfusion of the pig thyroid gland with blood containing high levels of calcium produced systemic hypocalcemia. Since there was no parathyroid tissue in the pig thyroid gland, this experiment clearly demonstrated that the factor responsible for the hypocalcemia resided in the

thyroid. Finally, it was shown that extracts of this gland produced a fall in plasma calcium.

It was probably Baber, in 1876, who first reported the existence of a different secreting cell in the thyroid and the extensive work of Nonidez (1932), which lead to its identification, should be mentioned. In the sixties, the calcitonin cells were identified and their secretory function well established as the result of work involving enzymatic and immunofluorescent techniques(Foster et al., 1964; Bussolati et al., 1967 and 1968). The calcitonin secreting cells were named the "C" cells by Pearse (1966) to indicate the association of this cell with the secretion of calcitonin. They have also been called the clear cells by Stux et al. (1961), because they stain pooly with the periodic acid Schiff reaction, parafollicular cell, mitochondrial rich cell, protoplasmic rich cell, ovoid cell, basal granular cell, basal follicular cell and interfollicular cell. In 1937, Godwin had suggested that the "C" cells were derived from the ultimobranchial body developing from the last pair of pharangeal pouches in the developing embryo. During the developing process, the ultimobranchial body containing the "C" cells migrates to the descending thyroid. Calcitonin has also been extracted from the parathyroid gland, and the thymus (Copp and Parkes,1968); as a consequence, it has been suggested that not all of the "C" cells migrate to the thyroid.

Structure of Calcitonin

In 1968, porcine calcitonin was isolated and the amino acid sequence was determined independently by Potts et al.; Neher et al.; and Bell et al.. This has been subsequently accomplished for ovine, bovine, human and salmon

calcitonin. It is now possible to obtain the synthetic, biologically active calcitonin hormone. The molecule of salmon calcitonin used in our experiment is a straight chain peptide of 32 residues with a proliamide in place of a free carboxyl group at the C terminal and a disulfide bond linking residues 1 to 7. It has few charged side chains, a relatively large number of hydrophilic side chains and the overall molecule has a lipophilic character. Salmon calcitonin has been reported to be 25 to 50 fold more potent in every mammalian species tested than any other mammalian calcitonin.

Action

The principale stimulus for the secretion of calcitonin is calcium but it can also be affected by factors such as other hormones, antibodies, and possibly magnesium (Bell, 1970). Evidence indicated that the hormone may function primarily as an emergency mechanism to control dangerous hypercalcemia. This is suggested by the results of experiments by Hirsh et al. (1964) where a prompt fall in both plasma calcium and plasma phosphate level was registered, following the injection or infusion of calcitonin into normal rats.

Since the lowering in serum calcium concentration could be the result of a decreased entry or an increased removal of these ions in the blood, therefore, the tissues of the body which have received the most attention as targets for calcitonin are bone, because it is the site of entrance of exogenous calcium, and the kidney because of its excretory function.

Hard tissue Mazzuoli et al. (1966), Aer (1968), and Johnston et al. (1966), showed that calcitonin reduces the urinary excretion of radio-

active bone tracer elements, such as ^{85}Sr and ^{45}Ca, previously incorporated into bone. They also reported an inhibitory effect of thyrocalcitonin on calcium release *in vivo* and bone metabolism *in vitro*. In 1967, MacIntyre et al. perfused a cat limb with the hypocalcemic hormone and reported an arteriovenous difference in plasma calcium concentration. The action of calcitonin on resorption of bone has been further substantiated by histologic studies carried out in rat and mouse bones by Foster et al. (1966 and 1967). In their experiment, the examination of bone tissue from animals who had received calcitonin *in vivo* indicated that the hormone induced morphological changes in osteoclasts. Furthermore, the number of osteoclasts was greatly reduced when compared with the amount found in non-injected animals.

Marx et al. (1972) and Goltzman (1980) using cell membrane preparations derived from fetal rat or rabbit calvaria and tibia, reported significant raises in cAMP after addition of calcitonin to the preparation. Their results suggested the presence of receptors for the polypeptide hormone. Moreover, Marx and Goltzman's studies were confirmed by *in vitro* specific binding assay using radioiodinated salmon calcitonin which verified the presence of saturable sites for the hormone in their cell membrane preparation. All of the above provide enough evidence to support the general opinion that calcitonin acts by decreasing the rate of bone resorption.

The possibility that calcitonin might affect bone formation has been investigated, but the results are often conflicting.

Soft tissue The studies done on the gastrointestinal tract suggested that the calcium lowering action is not achieved through this system. This

was shown by Munson et al. (1966), who demonstrated that calcitonin lowered serum calcium in the rat, even after removal of the intestinal tract. The additional study by Krawitt (1967) showed that calcitonin did not affect absorption of calcium from isolated loops of the intestine of rats.

The literature on the role of the kidney in the hypocalcemic response of calcitonin is controversial. Calcitonin has been reported as bringing about phosphaturia (Robinson et al., 1967) and also as a hormone capable of decreasing the urinary excretion of phosphate (Hirsch et al., 1964). In 1969, Pak et al. demonstrated that calcitonin increased renal clearance of calcium in thyroparathyroidectomized dogs. More recent data from experiments carried out by Marx et al. (1972 and 1973), and Goltzman (1980), involving activation of adenylate cyclase and *in vitro* specific binding assay using radiolabeled calcitonin, strongly suggested the presence of specific receptors for calcitonin in kidneys.

MATERIAL AND METHODS

MATERIAL AND METHODS

Several experiments have been carried out in order to determine the *in vivo* specific binding for the synthetic polypeptide hormone salmon calcitonin (sCT) and to establish the exact identity of its target cells.

The present thesis will describe the results obtained from three experiments referred to as *calcitonin 1*, *calcitonin 2* and *calcitonin 3*. These experiments consist of straightforward *in vivo* specific binding determination with a slight modification in *calcitonin 3* experiment, in which the animals were injected with equal doses of tritiated thymidine (^{3}H-tdr) one hour prior to the actual injection of the salmon calcitonin. In *calcitonin-bPTH* experiment, instead of cold sCT, a different peptide, cold bovine parathyroid hormone (bPTH (1-34)), was used to verify the specificity of the binding. A fifth experiment, referred to as *calcitonin-Ca lowering activity*, was designed to test the biological integrity of the iodinated sCT molecule, as compared to the unlabeled synthetic molecule, by measuring their effect on plasma calcium levels after intravenous injection in rats.

IODINATION OF SYNTHETIC SALMON CALCITONIN

Synthetic salmon calcitonin (sCT); approximately 2500mU/μg, a generous gift of Sandoz Co. LTD., Basel, Switzerland, was iodinated with isotopic sodium iodide (Na ^{125}I); specific activity 17 μCi/mg, New England Nuclear, Boston, Mass., by the Chloramine T method described by Hunter and Greenwood, 1962.

The stepwise iodination process involves the following: the isotopic sodium iodide ($Na\ ^{125}I$) is first diluted with 0.05M phosphate buffer at pH 7.5 to a final solution containing 1 mCi of ^{125}I per 10 μl of 0.02M phosphate buffer at pH 7.5. An appropriate amount of chloramine T (sodium salt of N-monochloro-p-toluene sulfonamide) is added to an equimolar solution of $Na\ ^{125}I$ and sCT.

The chloramine T is a mild oxidizing agent which slowly liberates hypochlorous acid in aqueous solution. The exact mechanism of the iodination reaction is not known but presumably a complex of iodide with the sulfonamide is formed in which the iodide carries a positive charge. It is thought that by means of an electrophilic substitution, the iodide-sulfonamide complex reacts with the six carbons ring of the tyrosyl residue in position twenty two of the salmon calcitonin molecule. The iodination is carefully timed for a duration of 30 seconds to avoid potential radioactive damage and to prevent sur-iodination. The reaction is terminated with the addition of sodium metabisulfite which quenches the ionization effect of the chloramine T, therefore preventing further iodination.

A sample of the preparation is used to determine the percent incorporation of the isotopic iodide. Trichloroacetic acid (TCA) is added to precipitate the iodinated sCT, and a gamma scintillation counter (Packard model 3002), is used to determine the total cpm and cpm contained in the pellet. If the percent incorporation is judged satisfactory, the iodinated hormone is precipitated by the addition of QUSO G-32 (microfine precipitated silica, Quartz. Co.) in a SEMP solution (0.2M phosphate buffer ph 7.0, 1.2M NaCl, 0.05M EDTA, 0.01% merthiolate). The mixture is spun at 6000 rpm for approximately two minutes (table top centrifuge 6). After this step, the supernatant, containing free iodide, protein fragments and

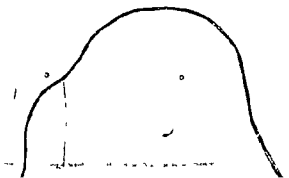
unlabeled sCT, is discarded.

Addition of Anion Exchange Resin (AG 1-X10, 200-400 mesh chloride form, Bio Rad laboratories, Richmond, California) removes any iodide. This is followed by the addition of a mixture of 20% (v/v) acetone and 1% (v/v) acetic acid in order to separate the iodinated sCT from the QUSO. This mixture is centrifuged at 6000 rpm for two minutes. The supernatant containing the ^{125}I-sCT is subsequently evaporated to dryness under a stream of nitrogen and redissolved in 2.5% (w/v) bovine serum albumin in 25mM TRIS-HCl buffer at pH 7.4. Iodination was accomplished immediately before each experiment described. After iodination each batch was subsequently divided into 2 portions, one portion was injected into the experimental animal and the second was used to prepare the material injected into the control animal. In this way, the exact amount of radioactive hormone, with the same specific activity was injected into both animals.

ASSESSMENT OF THE BIOLOGICAL ACTIVITY OF THE IODINATED SALMON CALCITONIN

The biological activity of identically labeled hormone has been confirmed by three separate procedures. The stimulation of adenylate cyclase and the *in vitro* binding assay, have been previously reported. As part of this work, the *in vivo* plasma calcium determination was used to assess the biological hypocalcemic activity of the iodinated salmon calcitonin.

Adenylate Cyclase Stimulation Study. Although not a part of the experiments done for this thesis, the methodology is given for the sake of completion. It is currently accepted that polypeptide hormones bind to receptors situated on the plasmalemma of their target cells. The binding of the hormone with its receptor causes the formation of an intracellular



messenger molecule that stimulates some characteristic biochemical activity of the target cells which bring about the specific biological response. It has been reported that binding of calcitonin to receptors activates the membrane bound adenylate cyclase which is responsible for the conversion of ATP into cAMP. As a consequence, if the hormone is added to a membrane preparation in the presence of ATP, an increase in cAMP is interpreted as indirect evidence of biological activity.

Salmon calcitonin-adenylate cyclase studies involve the preparation of highly purified plasmalemma preparation from homogenates of renal tissue and fetal rat calvaria. The homogenates can be fractionated by centrifugation on sucrose gradients to obtain a fraction relatively free of other cell components. This is indicated by a decrease in specific activity of marker enzyme for the contaminants and an increase of marker enzyme for the plasmalemma . Electron microscopy of kidney plasmalemma preparation has shown that the purified membrane fractions contain mitochondria and fragments of brush border (Mark, Fedak and Aurbach, 1973) indicating a low degree of contamination. Based on the above work, the biological activity of the synthetic salmon calcitonin (sCT) and that of the iodinated sCT has been assessed through comparative studies. The concentration of cAMP has been determined after addition of increasing amounts of sCT and of ^{125}I-sCT. Results showed a linear increase in cAMP proportional to the amount of calcitonin added to the preparation. Moreover, the overlapping of the graphs obtained from both the unlabeled and the labeled molecule indicated that the iodinated species is indistinguishable from the non-iodinated calcitonin in activating the rat renal and skeletal adenylate cyclase enzyme (Mark et al., 1972).

In Vitro Competitive Binding Assay. In this type of experiment, a series of salmon calcitonin and salmon calcitonin analogs are tested against the iodinated salmon calcitonin for uptake by highly purified skeletal and renal plasmalemma preparations. After a proper time of incubation, bound hormone is separated from unbound hormone and the uptake of radioactivity is determined with a liquid scintillation counter. The results of these competitive experiments are expressed as Dose Response curves. Results indicated that only the cold salmon calcitonin could compete with the labeled molecule to bind the receptors in the membrane preparation (Mark et al., 1972; Goltzman, 1980).

In this type of assay, the binding capacity of the iodinated species is expressed as a percent of the total radioactivity added to the preparation. The fact that the sCT, but not the analogs, can compete with the ^{125}I-sCT is another way to verify the similarity between the iodinated and the non-iodinated species and an indirect way of assessing the biological activity.

In Vivo Plasma Calcium Determination after Injection of ^{125}I-sCT and sCT

It has already been demonstrated, (Parsons and Reynolds, 1968), that the iodinated salmon calcitonin when injected intravenously caused a decrease in plasma calcium concentration. In order to verify the *in vivo* biological activity of the labeled species used in our experiments, duplicate male Sherman rats anaesthetized with .1% (v/w) of Nembutal were injected intravenously with either iodinated ^{125}I-sCT (specific activity 710 μCi/μg; 50x10^6 dpm or 500x10^6 cpm) or unlabeled sCT (.03 μg or .3 μg) or buffer alone (2.5% (w/v) bovine serum albumin in 25mM Tris-HCL, pH 7.4). One hour after injection, the animals were exsanguinated through the dorsal aorta. The collected blood samples were centrifuged and the calcium concentration

in the plasma fraction was analysed, using a sequential Multiple Analyser[18] with Computer (Technicon Inc.) following the method of Gitelman (1967).

The animals injected with buffer alone, served as control and showed a plasma calcium concentration of 9.5 mg/dl. The injection of a dose of ^{125}I-sCT containing ($50x10^6$ dpm) showed a significant fall in plasma calcium from 9.5 mg/dl to 8.2 mg/dl and was paralleled by the fall obtained when .03 μg of sCT was injected. When a ten fold larger dose of ^{125}I-sCT was injected ($500x10^6$ dpm), a greater fall in plasma calcium from 9.5 mg/dl to 7.5 mg/dl was recorded and was also paralleled by a similar decrease produced after the injection of .3 μg of the cold sCT. These results clearly demonstrated that the synthetic salmon calcitonin is biologically active and that the iodinated form is indistinguishable from the non-iodinated molecule in its biological activity.

ANIMAL PROCEDURES

For the experiments performed, all animals and tissues have been treated similarly with few exceptions. In *Calcitonin* 3 one hour prior to the injection of the radioactive hormonal preparation, both the experimental and the control animals were injected intraperitoneally with .25 ml of tritiated thymidine in aqueous solution (^{3}H-tdr; 1.0mCi/ml; New England Nuclear, Boston, Mass.). In *Calcitonin-bPTH*, a second control animal was injected with the normal radiolabeled preparation mixed this time which an excess of 125 μg of bovine parathyroid hormone (bPTH; 6000mU/μg; Beckman, Palo Alto, California).

Five minutes prior to the beginning of the experiment, male Sherman rats weighing 100±10 g received an intraperitoneal injection of .1% (v/w) Nembutal. Under mild anaesthesia, the animals were injected through the

external jugular vein with the appropriate radioiodinated preparation (Table I). Once injected, the iodinated preparation was allowed to circulate 2.5±.5 minutes. Animals were sacrificed by gravity perfusion 12±2 ml/10 sec) through the left ventricle, initiated with a lactated Ringer's solution (Abbot Laboratories) for 25±5 seconds in order to wash out the unbound and circulating hormone. This was immediately followed by 2.5% glutaraldehyde in 0.05M Sorensen's phosphate buffer containing 0.1% (w/v) sucrose at pH 7.3 for 20±10 minutes. At the end of the perfusion, the tissues of interest were dissected and left over night in the same fixative at 4^0C.

TISSUE PREPARATION

Paraffin Embedding

After glutaraldehyde perfusion-fixation, the entire brain was dissected and further trimmed by removing the tissue just anterior to the optic chiasm and the tissue just posterior to the median eminence by coronal cuts with a razor blade. The dissected brains were post-fixed in Bouin's Fluid for a period of 24 hours, washed in several changes of 50% (v/v) and 70% (v/v) alcohol to remove the Bouin's Fluid and stored in 70% (v/v) alcohol solution. Dehydration was achieved in four changes of 100% dioxane spread over a period of 24 hours, followed by another 24 hours of infiltration in four changes of 100% paraffin at 56^0C. The infiltrated brains were embedded in blocks of paraffin in such a way as to obtain serial cross sections of 4 µm thick starting anteriorly at the level of the optic chiasm. Sections were cut with a spencer A/O microtome, put on glass slides, and left on a hot plate at 47^0C to allow then to stretch and dry. After deparaffinization in xylene and rehydration in baths of decreasing alcohol concen-

tration until distilled water, the slides were immersed for ten minutes in hematoxylin, washed in tap water, stained with Eosin for three minutes, rinsed in tap and distilled water and finally dried before processing for radioautography.

Epon Embedding

Prior to and after post-fixation, the tissues were washed in 6 changes of 10 minutes each with 0.5M phosphate buffer, pH 7.3. For post-fixation of the soft tissues, including liver, kidney and brain, a solution of 3% potassium ferrocyanide - 2% osmium tetroxide (Karnowsky, 1971) was used for a period of 1.5 hour at 4^0C. Alcoholic dehydration was achieved by increasing concentrations ranging from 70% (v/v) up to 100% (v/v) ethanol. Infiltration was initiated by 3 washes of 10 minutes each with propylene oxide followed by consecutive periods of 12 hours in solutions of increasing concentrations of Epon 812 in propylene oxide. After a final 12 hours in pure Epon, the tissues were encapsulated and polymerized in an oven for 48 hours at 60^0C.

After polymerization, the blocks were trimmed and 0.5 µm or 1.0 µm thick sections were cut with glass knives on a Huxley ultra-microtome for light microscope radioautography. The sections were placed on glass slides and dried on a hot plate at 80^0C.

For pre-staining with iron hematoxylin, the slides were placed on a hot plate prewarmed to 80^0C, flooded with a mordant solution of 5% (w/v) ammonium sulfate for an appropriate amount of time, rinsed in distilled water and replaced on the hot plate. In a second step, they were flooded with Regaud's hematoxylin (1% (w/v) hematoxylin;

10% (v/v) ethyl alcohol, 10% (v/v) glycerine) for a period equal to that used for the mordant, rinsed again in distilled water, replacedon the hot plate, differentiated with tap water for three minutes followed by a last rinse in distilled water and dried on the same hot plate.

For electron microscope radioautography, the blocks were trimmed to the area of interest and silver-gold interference colour sections were cut on the same microtome with a diamond knife. The sections were positioned on glass slides previously coated with a solution of 1% (v/v) celloidin in isopentyl acetate. Prior to the radioautographic process, the sections were carbon coated, a step which facilitates the emulsion coating.

The mineralized tissues, including upper and lower incisors, humerus, and tibia were decalcified in 4.13% (w/v) disodium EDTA for 25±10 days at 4°C, a technique described by Warshawsky and Moore (1967). Once decalcified, the teeth were sectioned transversely into four segments, then each segment was cut into two pieces along the longitudinal axis. The humerus and tibia were first cut in the longitudinal axis and retrimmed to demonstrate the entire epiphyseal plate and surrounding tissue. The trimmed tissues were left overnight in the buffer washing solution (0.15M phosphate buffer, pH 7.3) at 4°C to remove any EDTA from the tissues. The tissues were then post-fixed, dehydrated, and infiltrated exactly as in the case of the soft tissues.

Radioautography Both the 4 μm thick paraffin sections and the 0.5 μm thick Epon sections were coated with Kodak NTB2 emulsion and exposed for various intervals (Kopriwa and Leblond, 1962). For electron microscope radioautography, sections were prepared according to Kopriwa (1973).

After exposure, the electron microscope radioautographs of the soft and hard tissues were transferred to Athene electron microscope grids.

The sections on the grids were stained for 10 minutes with Reynold's lead citrate stain (Reynolds, 1963) after treatment with either acetic acid for qualitative photomicrographs or isopentyl acetate for electron micrographs to be used for quantitation.

QUANTITATIVE ANALYSIS OF RADIOAUTOGRAPHS

Kidney

Silver grains were counted with the light microscope over proximal convoluted tubules (TableII) and over distal convoluted tubules (Table VI) in the cortex of the kidneys. Similarly, but in the outer medulla of the kidneys, the counts were made over the descending limb of the loop of Henle (Table III), thin limb of the loop of Henle (Table IV), and ascending limb of the loop of Henle (Table V). Counts were obtained from similarly exposed radioautographs from the experimental and from the control animals. The diameter of each tubule was measured in two axis at right angles to each other. The average diameters were calculated and compared among several rats in experimental and control kidneys. Since no statistically significant size differences were found, the counts were expressed as grains per cross sectioned tubules (Table II - TableVI).

Bone

For quantitation with the light microscope, two analyses were performed over similarly exposed radioautographs from the experimental and control animals. First, grains were counted over the tissue at the epiphyseal surface of the growth plate of tibia in areas of 3,000 μm^2 delineated by an ocular micrometer grid (Table XI). Second, the grains were counted over clearly defined multinucleated cells of an approximately equal size of

1200±200 μm^2 as determined with an ocular micrometer grid. The counts were made over osteoclasts from the calvaria (Table VII), from the alveolar bone around the rat upper incisors (Table VIII), from the metaphyseal surface of the proximal growth plate of the humerus (Table IX) and the tibia (Table X). The silver grains related to osteoclasts in electron microscope radioautographs were estimated from micrographs at a magnification of 30,000 X. Grains were scored as either within the osteoclasts or outside the osteoclasts, or directly on the plasma membrane (or within a radius of 230 nm from the grain center).

RESULTS

RESULTS

RADIOAUTOGRAPHIC LOCALIZATION OF IODINATED SALMON CALCITONIN (^{125}I-sCT)

Kidney

An intense radioautographic reaction was observed over the proximal convoluted tubules in the cortex from both the experimental (Fig. 1) and control (Fig. 2) rats. Within the tubules, the silver grains appeared to be located predominantly at the base of the brush border (Figs. 1 and 2), that is, at the luminal aspect of the proximal convoluted tubules. With electron microscopy (Figs. 3 and 4), the filamentous radioautographic silver grains were localized at the base of adjacent microvilli, mainly over small coated vesicles or at the periphery of larger vacuoles in the area between the brush border and the supranuclear cytoplasm. When the grains were found in the supranuclear region, they were associated with dense bodies presumably of lysosomal origin. Since the distribution of grains over the proximal convoluted tubules was identical in experimental and control animals and no quantitative decrease could be shown in the number of silver grains per cross sectioned tubule in the control animal of *calcitonin 1*, *calcitonin 2* and *calcitonin 3* experiments (Table II), it was concluded that the reaction represented binding of labeled hormone to low affinity, high capacity nonspecific binding sites. These sites are probably related to resorption and subsequent degradation of labeled hormone from urinary filtrate. This interpretation has been substantiated by the results obtained after injection with other labeled polypeptides such as ^{125}I-insulin,

^{125}I-prolactin and ^{125}I-parathyroid hormone. In all cases the results showed the exact same reaction distribution. When Na ^{125}I was injected, no silver grains were found in relation to the vacuoles of the apical portion of the proximal convoluted tubule cells, thus demonstrating that radioautographic silver grains over the proximal convoluted tubules were not the results of resorption of free labeled iodide.

A moderate number of silver grains was present over the basal aspect of the distal convoluted tubules in the cortex of the kidney from experimental animals (Fig. 1). Electron microscope radioautography showed that the silver grains were localized to the highly infolded basal cell membrane (Figs. 5 and 6). Grains were almost completely absent over the control kidney (Fig. 2). Grain counts per cross-sectioned distal convoluted tubule obtained from radioautographs of experimental and control animals of *calcitonin 1*, *calcitonin 2*, *calcitonin 3* and *calcitonin-bPTH* experiments showed a decrease in counts ranging from 1.7 to 4.8 fold (Table VI). Thus, these basally disposed grains along the distal convoluted tubules represented specific, high affinity and low capacity binding sites for calcitonin.

In addition, the ascending limb of the loop of Henle in the outer medulla, demonstrated radioautographic reactions on the basal aspect of cross sectioned tubules. Electron microscope radioautography showed that the silver grains were localized to the moderately infolded basal cell membrane. Grain counts per cross-section of this portion of the uriniferous tubule obtained from *calcitonin 1*, *calcitonin 2*, *calcitonin 3*, and *calcitonin-bPTH* experiments (Table V), showed a marked decrease in counts ranging from 2.7 to 5.2 fold. Thus, these basally disposed grains along the ascending limb of the loop of Henle also represented high affinity low capacity

specific binding sites for calcitonin.

A very light radioautographic reaction was related to the basal aspect of the descending limb of the loop of Henle in the cortex and in the outer medulla of the kidney. Since the distribution of grains was identical in the experimental and in the control animals, and quantitative analysis did not show any significant decrease in counts per cross-sectioned tubules of *calcitonin 1*, *calcitonin 2*, *calcitonin 3* and *calcitonin-bPTH* experiments (Table III), it was concluded that these grains represented nonspecific binding. This was also true for the thin limb of the loop of Henle (Table IV).

In summary, only a limited portion of the uriniferous tubule has been shown to contain high affinity receptors for the hypocalcemic hormone ^{125}I-sCT. This portion consists of the segments of the uriniferous tubules identified as the ascending limb of the loop of Henle and the distal convoluted tubule.

Bone

Bone tissue obtained from the calvaria, alveolar bone of the maxilla, and from the epiphyseal plates of the humerus and the tibia were examined. In experimental animals (Figs. 7, 9, 11, 13, 16, and 19) prominent labeling was present over multinucleated osteoclasts and over mononuclear cells and cell fragments of uncertain identity. However, clearly identifiable osteoblasts, osteocytes, chondrocytes and bone marrow cells were unlabeled. In control animals, no grains were found over any cell types (Figs. 8, 10, 12, 14, 17, and 20). Osteoclasts, from calvaria (Fig. 13), alveolar bone (Fig. 16), zone of vascular invasion of the humerus (Fig. 19) and tibia (Fig. 9), and in the area of the bone at the epiphyseal surface of the cartilage plate

of the tibia (Fig. 7), were heavily labeled predominantly at their periphery. Quantitative analysis was done on two regions of the tibia in light microscope radioautographs of *calcitonin 1*, *calcitonin 2*, and *calcitonin 3* experiments. Grain counts per unit area of the tissue on the epiphyseal surface of the growth plate showed that, in the experimental rats, the counts were significantly higher than in the control (Table XI). Grain counts over the individual osteoclasts from calvaria (Table III), alveolar bone (Table VIII), epiphyseal plate of humerus (Table IX) and tibia (Table X) confirmed that, in the experimental rats, the cells were significantly more labeled than in controls. The control values differed by factors ranging from 2.4 to 9.1 times. It was concluded that the grains on the cytoplasmic membrane of osteoclasts represent specific binding to high affinity, low capacity receptor sites for calcitonin.

Electron microscope examination of the cell fragments demonstrating labeling permitted them to be identified as portions of osteoclasts. The morphological features included multiple nuclei, an abundance of mitochondria, a sparcity of rER, and clusters of ribosomes, lysosomes and vesicles containing hydrolytic enzymes. The cells often showed the characteristic ruffled border and a clear zone containing only granular material. With the electron microscope, most of the radioautographic grains were positioned on the cell membrane, either near the basal part of the cell, that portion away from the bone surface (Figs. 22 and 23), or in the region of vesicles and vacuoles (Figs. 24 and 25) or in the clear zone (Fig. 25) as well as over all of the cytoplasmic processes (Figs. 22, 23, and 24) of the osteoclasts. No silver grains were seen over the surface of the cell which is thrown into numerous complex folds producing the ruffled border of the active cell. In order to

verify the position of the silver grains, counts in electron micrographs were made by placing a 230nm radius resolution boundary circle around each grain and recording the structure within the circle. Of the 845 grains counted, 68.6% were related to the cell membrane of the osteoclasts. This value is similar to that previously shown by Bergeron et al. (1977), for insulin receptors on hepatocytes.

The *calcitonin 3* experiment was designed to determine the nature of the labeled "mononuclear cell" which was usually located in the connective tissue space between blood vessels and fully differentiated osteoblasts. In this experiment, both the experimental and the control animals were injected with ^{3}H-thymidine one hour prior to sacrifice. It had previously been shown by Young (1962) and Scott (1967) that one hour after an injection of ^{3}H-thymidine, nuclear labeling is invariably confined to a cell similarly located and assigned to a bone population classically described as osteoprogenitor cells. Osteoclast nuclei are never labeled at that time. In the present study, it was first assumed that the "mononuclear cells" labeled with calcitonin could either be osteoprogenitor cells because of their morphology and location or portions of osteoclasts which appeared mononucleated because of the plane of section. The latter interpretation was strenghtened by the lack of nuclear labeling of these cells with ^{3}H-thymidine in radioautographs at the light microscope (Figs 9, 10, 11, 12) and electron microscope levels.

RADIOAUTOGRAPHIC CONTROLS OF BINDING SPECIFICITY

Injection of an Excess of the Cold Non Analogous Bovine Parathyroid Hormone

When ^{125}I-sCT was injected with a large excess of unlabeled and unrela-

ted hormone as in the *calcitonin-bPTH* experiment, no change in distribution or number of silver grains was observed over any tissue when compared with radioautographs from rats injected with ^{125}I-sCT alone (Figs.13 to 21). Figure 13, 16 and 19 were obtained from experimental animals injected with the same amount of ^{125}I-sCT mixed with an excess of cold sCT. Finally, figures 15, 18 and 21 were also from a control animal which had been injected with the same amount of ^{125}I-sCT, mixed this time with an excess of the cold unrelated hormone bPTH. There is no qualitative difference between the radioautographs obtained from the experimental animal (Figs.13, 16 and 19) when compared with the ones obtained from the control animal injected with cold bPTH (Figs.15, 18 and 21). The bPTH did not compete with the ^{125}I-sCT for occupancy of the receptors, thus demonstrating the specificity of the binding. This was substantiated by grain counts recorded in Tables II, III, V, VI, VII, IX, and X where no significant decrease in binding of the ^{125}I-sCT was detected in the control bPTH animal.

Injection of Isotopic Sodium Iodide

Radioautographs from tissues of rats injected with Na^{125}I showed no silver grains over bone cells or basal aspect of the ascending limb of the loop of Henle or the distal convoluted tubule.

DISCUSSION

DISCUSSION

Two major hormones, parathyroid hormone and calcitonin, are known to have opposing effects on the level of serum calcium (Milhaud and Moukhtar, 1966) and consequently on the regulation of bone homeostasis. In this study, ^{125}Iodide labeled salmon calcitonin was prepared utilizing a method known to preserve its biological activity (Marx et al., 1972 and 1973; Goltzman, 1980). Indeed, intravenous injection of this hormone depressed the plasma calcium level in a dose dependent manner which was identical to that given by the unlabeled hormone (Fig. 29). This labeled, biologically active hormone was used in a radioautographic study to localize the *in vivo* binding sites for calcitonin in suspected target tissues involved in calcium regulation. In control animals, the simultaneous administration of an excess of unlabeled calcitonin, but not of unrelated hormone produced a competitive inhibition of the specific saturable sites. Consequently, a comparison of radioautographs obtained from the experimental animal with the ones obtained from the control animal permitted the determination of specific binding sites in accordance with principles similar to the *in vitro* receptor binding studies. The outstanding advantages in the present method are the use of an *in vivo* system where none of the normal physiologic processes are disturbed, and the use of radioautography to locate the source of radioactivity. This technique allows for the resolution of labeling to individual, histologically identifiable cell types in any target tissue, including bone.

Identical results were obtained with ^{125}I-sCT in separate procedures involving four experimental and four control rats. The presence of radioactivity in all animals was confirmed by the nonspecific binding over the

proximal convoluted tubules seen in the kidneys of all experimental and control rats (Table II). Thus, the absence of radioactivity from specific binding sites in the control animals could be attributed only to competitive inhibition. In the experimental animals, the presence of labeling on identical cell types, in locations as remotely separated as calvaria, alveolar bone of the incisors, the humerus and the tibia, confirmed the reliability of the tracer methodology for specific binding sites. Parallel studies were designed to further assess the specificity of the binding. In the *Calcitonin-bPTH* experiment, the injection of the unlabeled parathyroid hormone with the labeled calcitonin did not reduce the number of silver grains over the specifically labeled cells of bone and kidney. However, grain accumulations were seen over the luminal aspect of the proximal convoluted tubules, similar to the nonspecific binding shown in the accompanying experimental animal of this experiment which had been injected with the labeled calcitonin. When labeled iodide was injected alone, no radioautographic grains were observed over the nonspecific binding sites of the proximal convoluted tubules, indicating that the nonspecific binding seen over these tubules is not due to the resorption of free iodide but to resorption of iodinated polypeptide molecules.

Because of the known effect of calcitonin, the presumed target tissues, bone and kidneys, were investigated together with a variety of other tissues in a search for any other possible sites of action.

Bone

No specific binding for ^{125}I-sCT has been shown on either osteocytes or osteoblasts. This seems to rule out the possibility of calcitonin being involved in osteocytic osteolysis, a process proposed by Bélanger (1969),

nor does it favor the hypothesis that hypocalcemia could be the result of an increased bone deposition by the osteoblasts.

Results showing specific binding of calcitonin by all osteoclasts of the body confirmed the data obtained from adenylate cyclase studies and *in vitro* specific binding studies. These studies have claimed that specific receptors for calcitonin are present in cellular or membrane preparations derived mostly from rabbit and rat tibia or calvaria. The disadvantage of these types of studies is that the preparations do not result in pure populations of cells and, therefore, it is impossible to attribute the binding to any given cell type. Thus, the results must often be expressed in very general terms.

Marx et al., (1972), have shown receptors for calcitonin in bone through biochemical studies involving the activation of adenylate cyclase and *in vitro* competitive binding assay with ^{125}I-sCT. In one study, (Rodan and Rodan, 1974), bone cells isolated from calvaria were treated with both thyrocalcitonin and parthyroid hormone. Because the effect of the two hormones was additive, it was postulated that these hormones acted on " bone cells " and presumably on separate sites. A more recent study (Goltzman, 1980), demonstrated an increase in cAMP in a preparation of membranes from cells of rat calvaria upon treatment with human calcitonin.

The above experiments are supported by the present work since specific binding sites for ^{125}I-sCT have been demonstrated on a bone cell identified as an osteoclast.

Additionally, the presence of specific sites for calcitonin related to this cell agrees with a study done on osteoclasts (Raisz, 1967) which showed that calcitonin inteferes with the action of parathyroid hormone by preventing the development of an extensive ruffled border. This is also substantiated by *in vitro* cell culture studies done by Kallio et al. (1971), who found striking changes in the ruffled border, loss of cytoplasmic coating from beneath the ruffled membrane and actual separation of the resorbing cells from the resorbing surface, after treating their cell cultures with calcitonin. From these data, they suggested that inhibition of bone resorption by calcitonin was directly related to a rapid response of the ruffled border and, as a consequence, their study supported the hypothesis that calcitonin acted by inhibiting osteoclastic bone resorption. They predicted that it was unlikely that the initial action of calcitonin was on the ruffled border, because of its close adherence to the bone surface, and it was possible that the binding of the hormone to receptors would be on those surfaces of the osteoclast which were exposed to the extracellular fluid. The actual localization of the binding sites as revealed in the present study confirmed the latter hypothesis.

Indeed, specific binding sites for ^{125}I-sCT were observed all over the cytoplasmic membrane of the osteoclasts with the exception of the membrane of the ruffled border. The membrane distribution of ^{125}I-sCT on the osteoclasts also supports the ultrastructural and histochemical results of Lucht (1973). He reported that after intravenous injection of calcitonin, osteoclasts showed an absence of ruffled border, an absence of phosphatase in the extracellular space between cell and bone, a decrease in the number of large vacuoles and no local accumulation of vacuoles in the cytoplasm. He also reported an increase in autophagosomes, all of which suggested inhibition of bone resorption by osteoclasts.

Taken together, these data allow us to propose a somewhat more elaborate hypothesis concerning the action of calcitonin on the skeletal system. It seems clear that calcitonin acts on bone by interfering with bone resorption. To do so, it would first bind to specific receptors on the surface of osteoclasts. As a result of the hormone-receptor binding, adenylate cyclase would be activated and would bring about a cellular increase in cAMP. This could indirectly interfere with the formation of the cytoplasmic coating beneath the ruffled border described by Kallio et al. (1971), and therefore lead to the disappearance of the ruffled border.

Kidney

This radioautographic study has shown specific receptors for calcitonin on a specific portion of the uriniferous tubule of the kidney, namely the ascending thick limb of the loop of Henle and the distal convoluted tubule. It is tempting to speculate that the presence of hormone receptors in this segment of the uriniferous tubule implies a functional role for calcitonin in the kidney. This disagrees with the generally held opinion that the major, if not the only, effect of calcitonin is that of inhibiting bone resorption. This supposition also disagrees with studies which could not assign an active role to the kidney in obtaining the hypocalcemic effect.

The present results, however, do support more recent data obtained from *in vitro* studies of adenylate cyclase activation and specific binding assay, which strongly suggest the presence of specific receptor sites for calcitonin in kidney membrane preparations (Mark et al., 1972 and 1973; Goltzman, 1980). Unfortunately, these experiments have been performed on kidney homogenates or on purified membrane fractions obtained from the cortex, outer or inner medulla of the kidney. Since each zone contains different functional segments of the nephron, it has been impossible to lo-

calize the precise site of binding of the hormone. The results reported in this thesis have been partially verified by a physiological study done by Chabardès et al., 1978. This study involved a microtechnique which allows enzymatic measurement on single, well-defined, segments of the nephron. Generally, their results showed no significantly consistent action of sCT at the proximal convoluted tubule, the descending and the thin limb of the loop of Henle in rabbit and mice. However, in mice, they reported a positive action on the ascending limb of the loop of Henle and on the distal convoluted tubule in all experiments.

Finally, Ardaillou (1978) used the *in vitro* specific binding assay for ^{125}I-sCT, coupled with adenylate cyclase stimulation, to show that calcitonin stimulated renal adenylate cyclase in some segments of the nephron, namely the thick limb of the loop of Henle and the initial part of the distal convoluted tubule. The results presented in this thesis agree with that distribution of specific binding sites for ^{125}I-sCT in the kidney.

In conclusion, the radioautographic localization *in vivo* of the receptors for the hypocalcemic hormone calcitonin has permitted the resolution of the controversy concerning the identity of the bone cell involved in the action of the hormone. It is clear from this study that calcitonin acts on the osteoclasts, most likely by inhibiting bone resorption, thereby contributing to lowering the amount of extracellular calcium available for entrance to the blood. This method has also identified the exact segment of the nephron acted upon by calcitonin, namely, the ascending thick limb of the loop of Henle and the distal convoluted tubule. Furthermore, the method has demonstrated for the first time that there are specific binding sites for calcitonin in the subfornical organ of the brain (Figs. 27 and 28).

The current opinion is that the hypocalcemia induced by calcitonin is achieved only through the action of calcitonin on bone. This idea has been weakened by the results of the present experiments.

Indeed, since specific binding for ^{125}I-sCT has been demonstrated in bone, kidney and brain, it is tempting to presume that all of these tissues are actively involved in the hypocalcemic effect of calcitonin. What remains to be determined is if they act in concert to bring about the calcium lowering response. However, the binding of hormone to tissues could equally mean that, in addition to, its hypocalcemic action, calcitonin may be involved in other physiologic processes that are still unknown.

TABLES

Table I Protocol of the five *in vivo* experiments with ^{125}I-salmon calcitonin in male Sherman rats

Experiment		Animal Weight in g	cpm X 10^6 injected per 100 μl	Specific Activity μCi/μg	μg "Hot" sCT	μg "cold" sCT
Calcitonin 1	Experimental	109	90	562.5	.144	--
	Control	108	70		.112	50
Calcitonin 2	Experimental	100	82	645	.119	--
	Control	110	81		.118	50
Calcitonin 3	Experimental*	96	255	712.5	.326	--
	Control*	94	255		.326	125
Calcitonin-bPTH	Experimental	92	200	737.5	.246	--
	Control(sCT)	84	200	737.5	.246	125
	Control(bPTH)	88	200	737.5	.246	--**
Calcitonin-Ca-lowering activity	Rat 1a,1b	90,90	23.4	712.5	.028	--
	Rat 2a,2b	75,80	234	712.5	.281	--
	Rat 3a,3b	80,80	--	--	--	.03
	Rat 4a,4b	84,78	--	--	--	.30
	Rat 5a,5b***	80,75	--	--	--	--

* These animals received 125 μCi of ^{3}H-thymidine (methyl ^{3}H ; New England Nuclear; Specific activity 20 Ci/mM) 1 hr prior to injection of calcitonin.

** The control animal received 125 μg of cold bovine parathyroid hormone (bPTH).

*** Normal rats injected with 2% BSA (bovine serum albumine-Tris 0.05M pH 7.5).

Table II Grain counts* per cross sectioned proximal convoluted tubules** in experimental and control rats injected with ^{125}I-Salmon Calcitonin

Experiment		Total grains	Mean grains per tubule	±SD	±SEM	P
Calcitonin 1	Experimental	4815	96	52	7.4	
	Control	5509	110	62	9.0	.2***
Calcitonin 2	Experimental	6662	113	37	5.2	
	Control	10583	213	55	7.6	.001***
Calcitonin 3	Experimental	7947	159	59	8.2	
	Control	13332	261	99	14.1	.001***
Calcitonin-bPTH	Experimental	22022	440	113	15.9	
	Control(sCT)	22446	448	117	16.6	.9***
	Control(bPTH)	14708	294	107	15.2	.001***

* Counted in light microscope radioautographs exposed for 7 days.

** 50 tubules counted in each rat.

*** In each experiment, the experimental value never exceeded the control except when bPTH was used to compete with the ^{125}I-sCT.

Table III Grain counts* per cross sectioned descending limb of the loop of Henle** in experimental and control rats injected with ^{125}I-sCT

Experiment		Total grains	Mean grains per tubule	±SD	±SEM	P
Calcitonin 1	Experimental	1013	20	11	1.6	
	Control	846	17	12	1.7	.2***
Calcitonin 2	Experimental	3416	68	20	2.9	
	Control	3277	66	18	2.6	.9***
Calcitonin 3	Experimental	518	10	7	1.0	
	Control	487	10	7	1.0	.8***
Calcitonin-bPTH	Experimental	783	15	10	1.4	
	Control(sCT)	910	18	9	8.8	.2***
	Control(bPTH)	734	14	6	6.2	.6***

* Counted in light microscope radioautographs.

** 50 tubules in each rat.

*** In each experiment, the experimental value never exceeded significantly the control value.

Table IV Grain counts* per cross sectioned thin limb of the loop of Henle** in experimental and control rats injected with ^{125}I-sCT

Experiment		Total grains	Mean grains per tubule	±SD	±SEM	P
Calcitonin 1	Experimental	103	2	2	0.3	
	Control	123	2	2	0.4	.9***
Calcitonin 2	Experimental	389	8	4	0.6	
	Control	570	11	7	1.0	.01***
Calcitonin 3	Experimental	171	3	3	0.4	
	Control	164	3	1	0.2	.8***

* Counted in light microscope radioautographs.

** 50 tubules in each rat.

*** In each experiment, the experimental value never exceeded the control value.

Table V Grain counts* per cross sectioned ascending limb of the loop of Henle** in experimental and control rats injected with ^{125}I-sCT.

Experiment		Total grains	Mean grains per tubule	±SD	±SEM	P
Calcitonin 1	Experimental	2093	42	16	2	
	Control	475	9	7	1	.001***
Calcitonin 2	Experimental	4370	87	27	4	
	Control	1667	33	18	2	.001***
Calcitonin 3	Experimental	815	21	8	1	
	Control	205	4	4	.6	.001***
Calcitonin-bPTH	Experimental	2868	57	18	3	
	Control(sCT)	598	12	12	.5	.001***
	Control(bPTH)	4580	92	92	3	.001***

* Counted in light microscope radioautographs.

** 50 tubules in each rat.

*** In each experiment, the experimental value exceeded the control value. When bPTH was used to compete with ^{125}I-sCT, the binding was increased by a factor of 2.

Table VI Grain counts* per cross sectioned distal convoluted tubules** in experimental and control rats injected with ^{125}I -Salmon Calcitonin

Experiment		Total grains	Mean grains per tubule	±SD	±SEM	P
Calcitonin 1	Experimental	1708	34	13.6	1.9	
	Control	382	7	7.4	1.1	.001***
Calcitonin 2	Experimental	2055	41	19.5	2.8	
	Control	1202	24	13.6	1.9	.001***
Calcitonin 3	Experimental	3072	61	28	4.0	
	Control	823	16	10	1.5	.001***
Calcitonin-bPTH	Experimental	8575	171	63	8.9	
	Control(sCT)	2029	41	14	2.0	.001***
	Control(bPTH)	8665	173	65	9.2	.9***

* Counted in light microscope radioautographs.

** 50 tubules counted in each rat.

*** In each experiment, the experimental value exceeded the control value except when cold bPTH was used to compete with the ^{125}I-sCT.

Table VII Grain counts* over osteoclasts** from the calvaria in experimental and control rats injected with ^{125}I-sCT

Experiment		Total grains****	Mean grains/osteoclast	±SD	±SEM	P
Calcitonin-bPTH	Experimental	4352	174	49	10	
	Control(sCT)	620	25	8	2	.001***
	Control(bPTH)	5052	202	58	12	.1***

* Counted in light microscope radioautographs.

** 25 osteoclasts selected for equal size of approximately 1000 μm^2.

*** The experimental value exceed the control by a factor of 6.9 but not when bPTH was used to compete with the ^{125}I-sCT.

**** All the grains over the cells counted in each rat.

Table VIII Grain counts* over osteoclasts** from the alveolar bone in experimental and control rats injected with ^{125}I-sCT

Experiment		Total grains	Mean grains/osteoclast****	±SD	±SEM	P
Calcitonin 2	Experimental	861	34	14	3	
	Control	332	13	6	1	.001***
Calcitonin 3	Experimental	1014	40	23	5	
	Control	318	13	13	1	.001***
Calcitonin-bPTH	Experimental	3265	131	26	5	
	Control(sCT)	542	22	9	2	.001***
	Control(bPTH)	5434	217	55	11	.001***

* Counted in light microscope radioautographs.

** A minimum of 25 osteoclasts selected for egual size of approximately 1000 μm^2.

*** The experimental value exceeded the control value by factors of 2.6, 3.0, and 5.9. When bPTH was used to compete with the ^{125}I-sCT, the binding of ^{125}I-sCT was increased by a factor of 2.

**** All of the grains over the cells counted in each rat.

Table IX Grain counts* over osteoclasts** at the metaphyseal surface of the proximal growth plate of the humerus in experimental and control animal injected with ^{125}I-sCT

Experiment		Total grains****	Mean grains/osteoclast	±SD	±SEM	P
Calcitonin 3	Experimental	1734	69	28	6	
	Control	181	7	6	1	.001***
Calcitonin-bPTH	Experimental	4905	196	61	12	
	Control(sCT)	814	33	19	4	.001***
	Control(bPTH)	4882	195	65	13	.8***

* Counted in light microscope radioautographs.

** A minimum of 25 osteoclasts selected for equal size of approximately 1000 μm^2.

*** The experimental value exceeded the control value by factorsof 9.1, 5.9 except when bPTH was used to compete with the ^{125}I-sCT.

**** All grains over the cells counted in each rat.

Table X Grain counts* over osteoclasts** at the metaphysis of the proximal tibial growth plate in experimental and control rats injected with ^{125}I -Salmon Calcitonin

Experiment		Total grains****	Mean grains/osteoclast	±SD	±SEM	P
Calcitonin 1	Experimental	2612	52	24.8	3.5	
	Control	966	19	12.2	1.7	.001***
Calcitonin 2	Experimental	2322	46	18.8	2.7	
	Control	968	19	8.4	1.2	.001***
Calcitonin 3	Experimental	4390	88	43.2	6.1	
	Control	651	13	9.4	1.3	.001***
Calcitonin-bPTH	Experimental	6990	280	99.9	20	
	Control(sCT)	1070	42	17.6	3.5	.001***
	Control(bPTH)	7320	293	53.9	11	.6***

* Counted in light microscope radioautographs.

** A minimum of 25 osteoclasts selected for egual size of approximately 1000 μm^2.

*** The experimental value exceeded the control value by factors of 2.7, 2.4, 6.8 and 6.9 respectively except when cold bPTH was used to compete with the ^{125}I-sCT.

**** All the grains counted over the cells in each rat.

Table XI Grain counts* over the tissue on the epiphyseal surface of the proximal tibial growth plate in experimental and control rats injected with ^{125}I - Salmon Calcitonin

Experiment		Total grains	Mean grains/3000 μm^2 **	±SD	±SEM	P
Calcitonin 1	Experimental	6588	132	55.7	7.9	
	Control	1342	27	15.0	2.1	.001***
Calcitonin 2	Experimental	4581	92	41.7	5.9	
	Control	2513	50	20.9	3.0	.001***
Calcitonin 3	Experimental	9862	197	82.8	11.7	
	Control	1047	21	10.5	1.5	.001***

* Counted in light microscope radioautographs.

** Grains were counted in a strip comprised of 50 rectangles layed across the epiphyseal surface of the plate. Each rectangle measured 100 μm long by 30 μm high. This value represents all the grains counted in the 50 rectangles.

*** The experimental value exceeded the control by factors of 4.9, 1.8 and 9.4, respectively in the 3 experiments.

FIGURES AND LEGENDS

FIGURE LEGEND ABBREVIATIONS

b	bone
bb	brush border
bl	basal lamina
bv	blood vessel
B	bone
CC	calcified cartilage
cart	cartilage
cp	cytoplasmic process
ct	connective tissue cell
cv	pinocytic coated vesicle
cz	clear zone
db	dense body
dct	distal convoluted tubule
en cp	endothelial cytoplasmic process
G	Golgi
L	lysosome
Lu	lumen
m	mitochondrion
ms	mixed spicule
n	nucleus
o	uncertain identity
ob	osteoblast
oc	osteoclast
ocy	osteocyte
pct	proximal convoluted tubule
rc	renal corpuscule
sL	secondary lysosome
va	vacuole

Figures 1 and 2 Kidney cortex from an experimental (Fig. 1) and a control animal (Fig. 2). Radioautographs of 0.5 μm thick Epon sections exposed 14 days. X 680.

Figure 1 Numerous silver grains are present over the cytoplasm of proximal convoluted tubules (pct). These grains are localized at the junction between the brush border and the apical cytoplasm. Distal convoluted tubules (dct) are labeled preferentially over their basal surface. Renal corpuscles (RC) are unlabeled.

Figure 2 Silver grains are present over identical locations within the proximal convoluted tubules (pct) of the control, thus indicating that the labeling is nonspecific. These heavy accumulations of silver grains presumably represent pinocytic uptake of labeled hormone from the urinary filtrate. Distal convoluted tubules (dct) are almost completely unlabeled indicating that the basal surfaces of these tubules possess specific binding sites for calcitonin. RC, renal corpuscle.

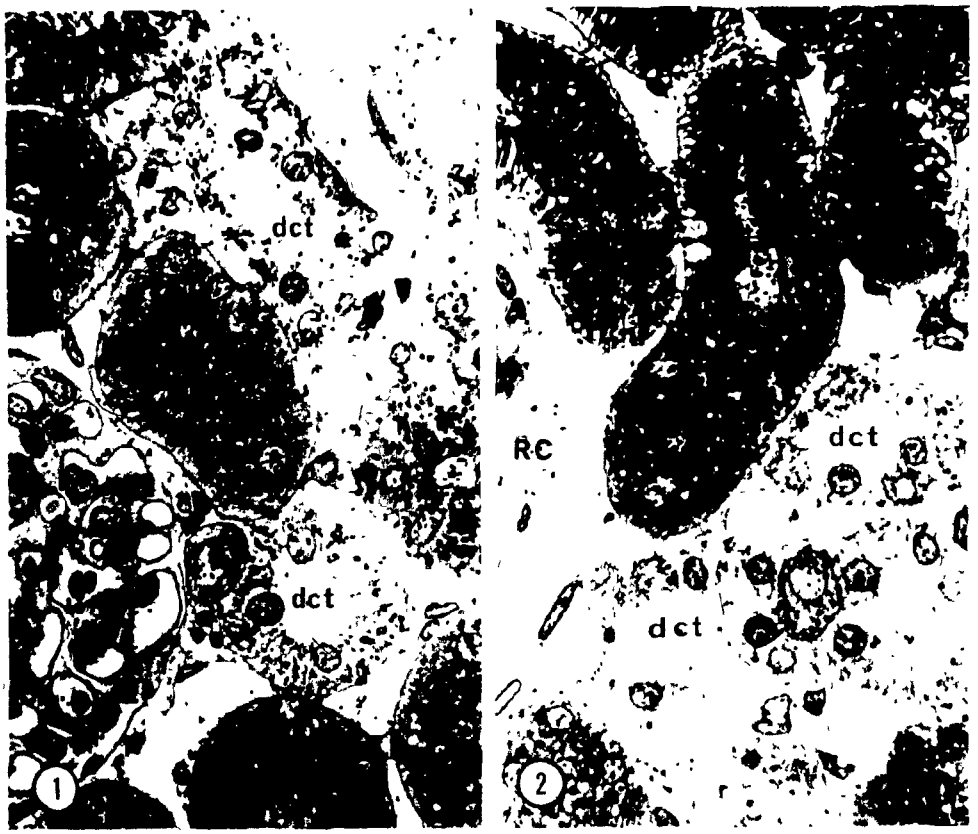
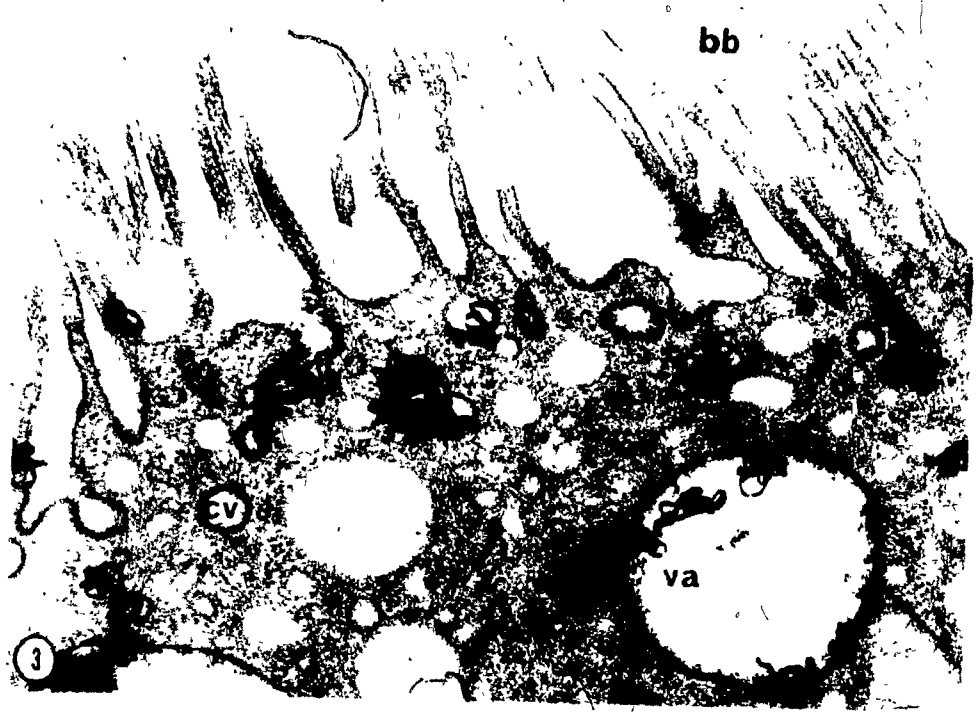

dct
dct
1
RC
dct
dct
2

Figure 3 and 4 Electron microscope radioautographs from kidney cortex of experimental animals.

Figure 3 Proximal convoluted tubule showing the location of the nonspecific labeling at the luminal aspect of the cells. The labeling is found at the base of adjacent microvilli of the brush border (bb), over small pinocytic coated vesicles (cv), or at the periphery of larger vacuoles (va). Exposed 80 days. X 30,000. m, mitochondria.

Figure 4 Proximal convoluted tubule showing a similar location of the nonspecific labeling. Again, grains are found over small vesicles (cv), and closely related to the periphery of larger vacuoles (va). Exposed 80 days. X 30,000. bb, brush border; m, mitochondria.


bb
cv
va
3


va
4

Figure 5 Electron micrograph of the basal aspect of the distal convoluted tubule (dct) from the cortex of an experimental animal showing silver grains associated with the deeply invaginated cell membrane. No silver grains were seen over the membrane of the proximal convoluted tubule (pct). The arrow heads indicate the basal lamina of the adjacent tubules. Exposed 80 days. X 30,000. en cp, endothelial cytoplasmic cell process; G, Golgi; m, mitochondria.

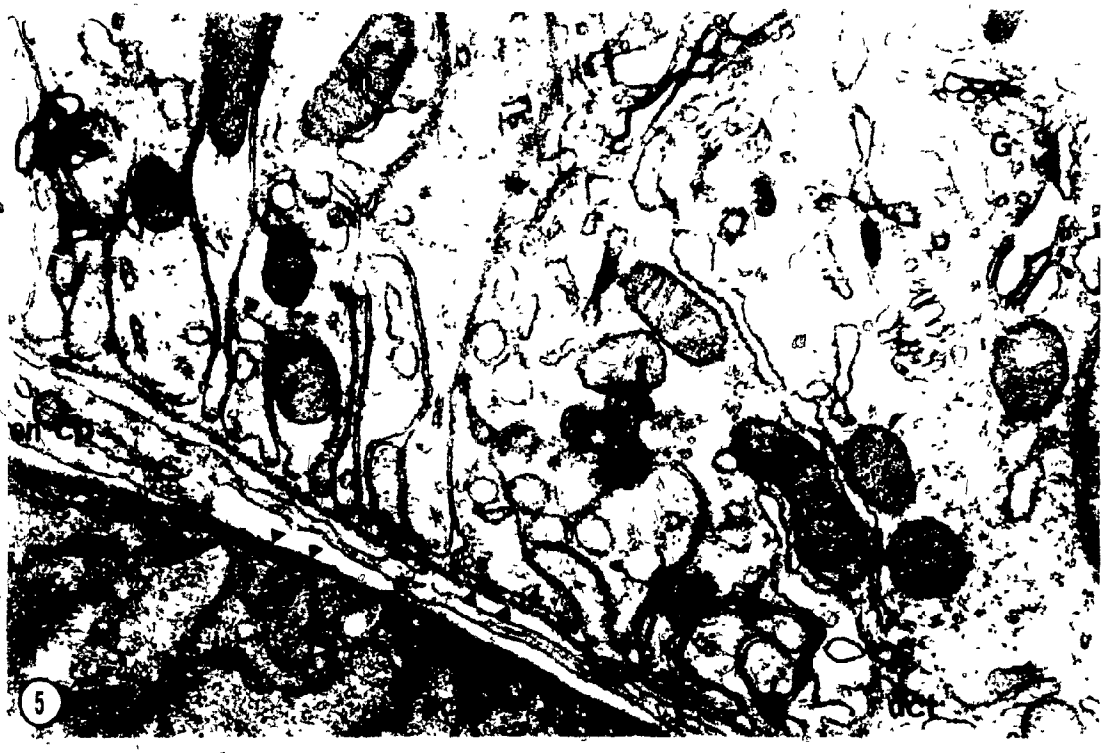

G
ert cp
5
UCT

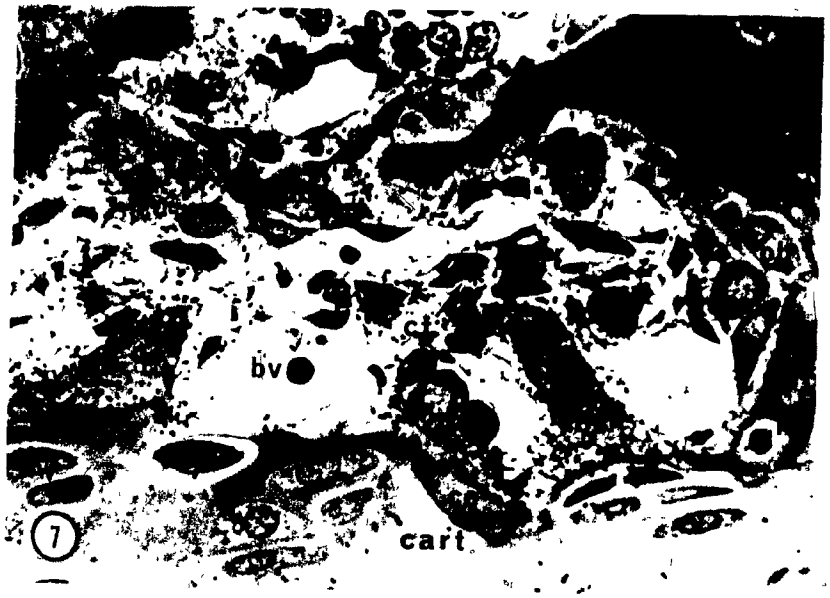
Figure 6 Electron micrograph of the basal aspect of the distal convoluted tubule from the cortex of the kidney of an experimental animal showing specific binding associated with the deeply invaginated cell membrane. The arrow heads indicate the basal lamina and the arrow, a tight junction between two adjacent tubular cells. Exposed 80 days. X 30,000. cp, cell process; db, dense body; Lu, lumen; m, mitochondria.


L
cp
m
6

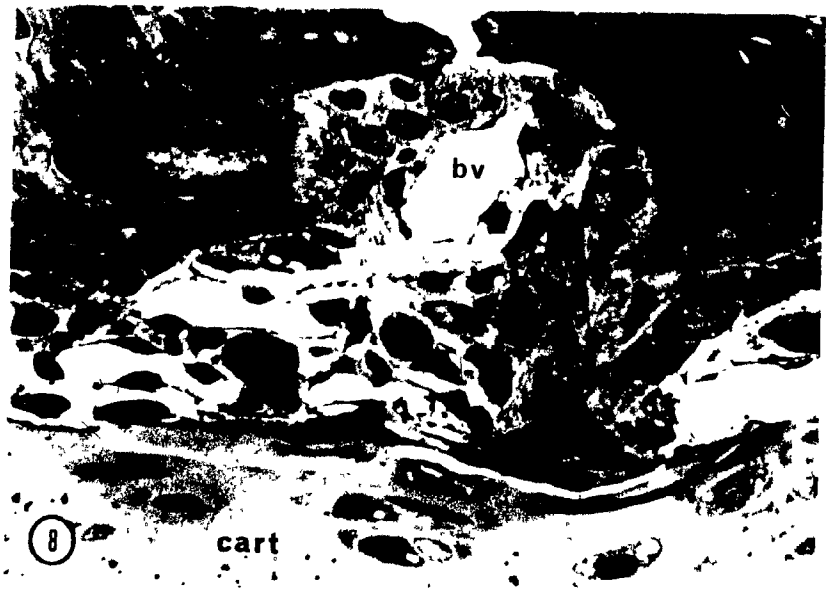
Figures 7 and 8 Epiphyseal surface of the cartilage growth plate in experimental (Fig. 7) and control animals (Fig. 8). Light microscope radioautographs of 0.5 μm thick Epon sections exposed 14 days. X 680.

Figure 7 The narrow space between the bone of the epiphyseal cavity and the cartilage of the growth plate (cart) contains capillaries (bv), osteoclasts (oc), osteoblasts (ob) and connective tissue cells (ct), some of which are presumed to be osteoprogenitor cells. In the experimental rat silver grains are found over osteoclasts (oc) and cells of uncertain identity (o). Electron microscopy revealed these to be fragments of labeled osteoclasts. Osteocytes seen within the bone, osteoblasts on the bone surfaces and chondrocytes within the cartilage plate are not labeled. The asterisk (*) indicates cells labeled with ^{3}H-thymidine injected one hour prior to labeled calcitonin. These cells, showing thymidine labeling, are not labeled by calcitonin and are presumed to be connective tissue cells (ct) with osteoprogenitor potential. cc, calcified cartilage.

Figure 8 Similar region in the control animal shows a scattering of silver grains which are not localized to any of the cell types. bv, blood vessel; cart, cartilage; ob, osteoblast; oc, osteoclast; ocy, osteocyte.



7



8

Figures 9 and 10 The zone of vascular invasion of the proximal tibial epiphyseal growth plate in experimental (Fig. 9) and control rats (Fig. 10). Light microscope radioautographs of 0.5 μm thick Epon sections exposed 14 days. X 680.

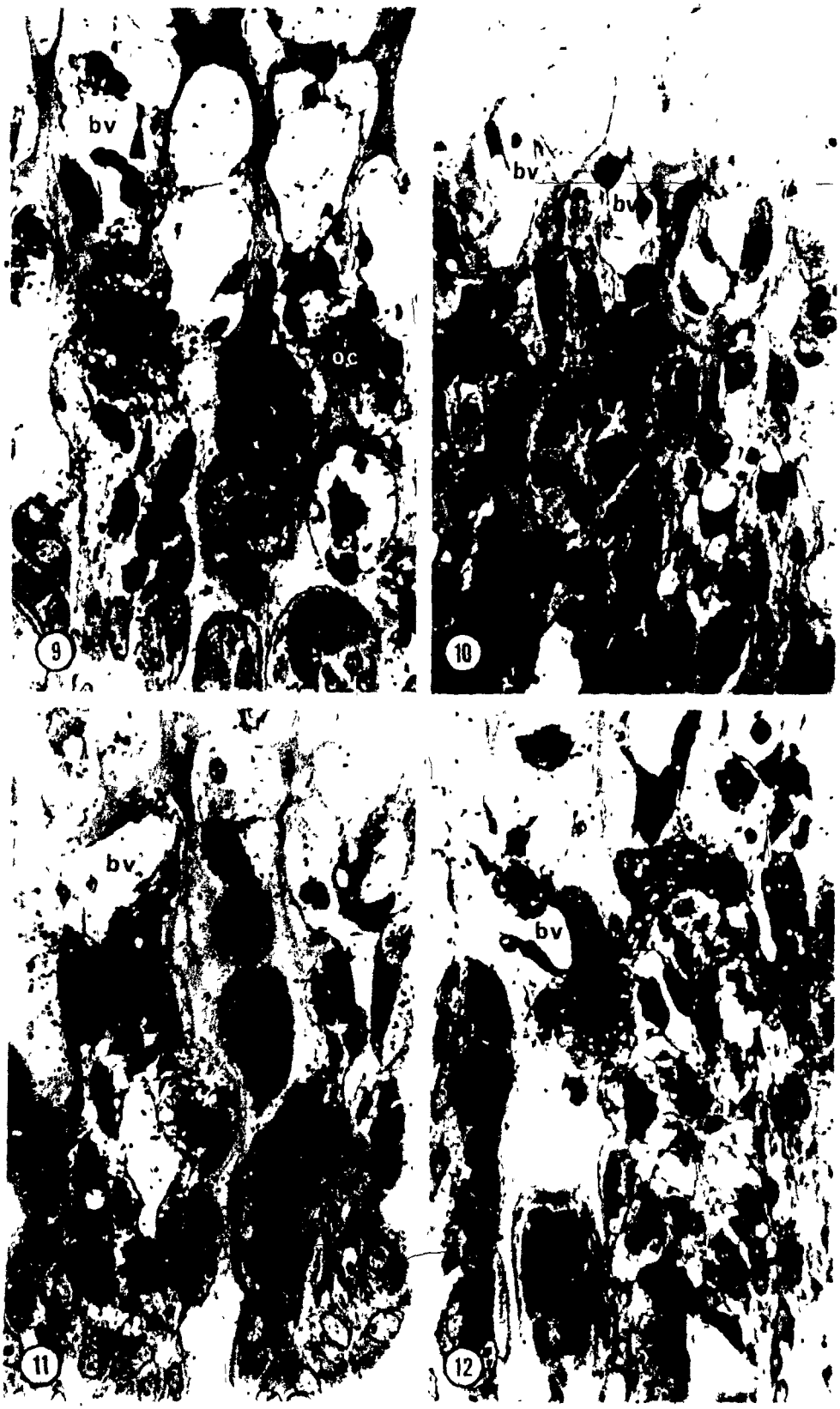
Figures 11 and 12 The zone of vascular invasion in the proximal humerus of experimental (Fig. 11) and control animals (Fig. 12). Light microscope radioautographs of 0.5 μm thick Epon sections exposed 14 days. X 680.

Figure 9 Below the zone of hypertrophic chondrocytes, osteoclasts (oc) are often seen along the remnants of calcified cartilage. These cells show numerous silver grains over the periphery of their cytoplasm. Connective tissue cells (ct), labeled with ^{3}H-thymidine (*) indicating their osteoprogenitor potential, are not labeled with ^{125}I-calcitonin. Fully differentiated osteoblasts (ob) are not labeled. bv, blood vessel associated with vascular invasion of chondrocyte lacunae.

Figure 10 Silver grains are absent over all structures in the section, except for ^{3}H-thymidine labeled nuclei of connective tissue cells presumed to be osteoprogenitor cells (*). bv, blood vessel.

Figure 11 Labeled osteoclasts (oc) are seen at the surfaces of calcified cartilage and mixed spicules. Osteoblasts (ob) are unlabeled. bv, blood vessel; *, ^{3}H-thymidine labeled cells.

Figure 12 Similarly located osteoclasts (oc) in the control animals are not labeled. Again, only ^{3}H-thymidine labeled cells (*) are seen. bv, blood vessel; ob, osteoblast.

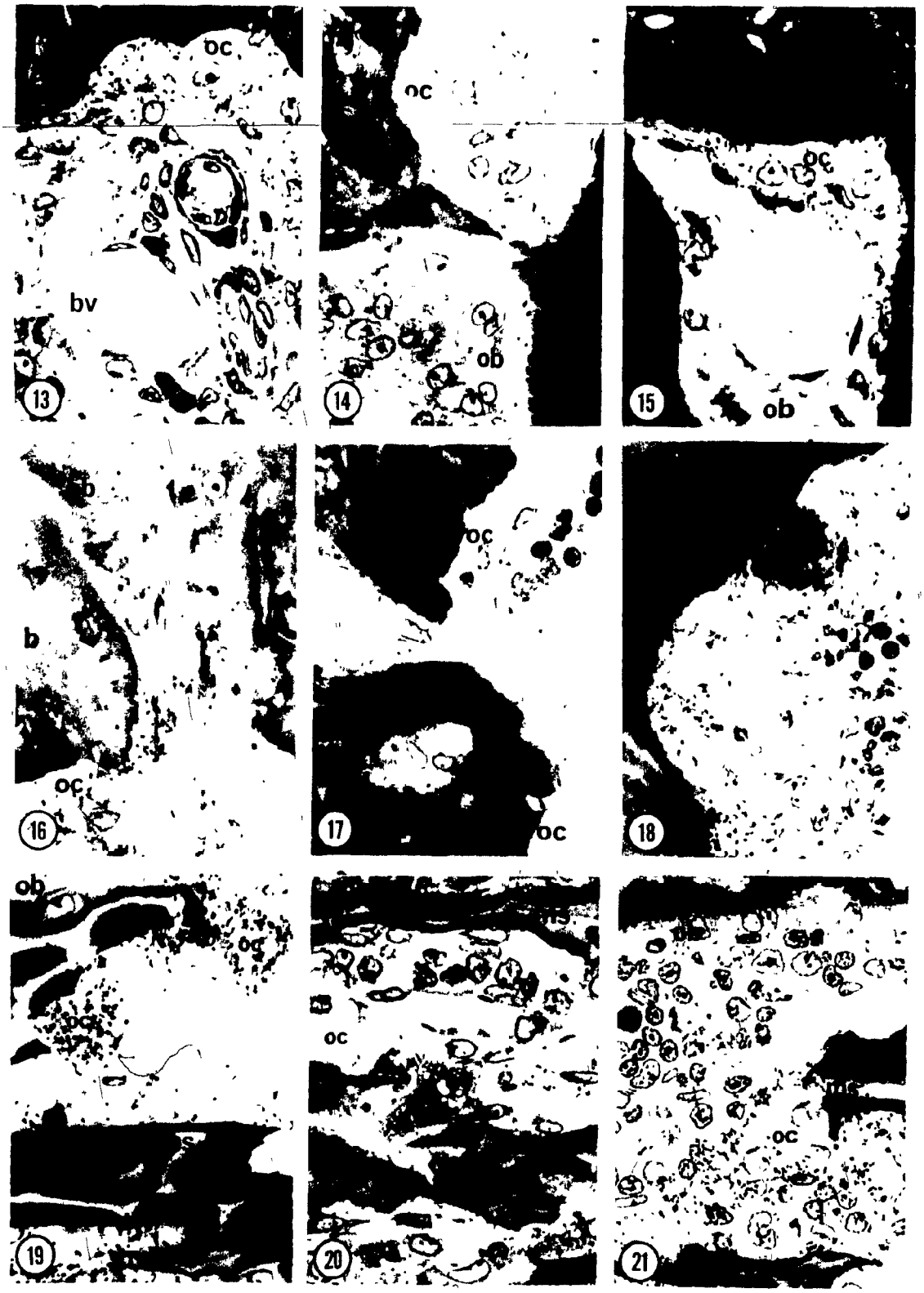

bv
oc
9
bv
bv
10
bv
11
bv
12

Figures 13-21 Light microscope radioautographs of 1 μm thick Epon sections from the calcitonin b-PTH(1-34) experiment. Exposed 7 days. X 600.

Figures 13, 16, and 19 obtained from the experimental animal; calvaria (Fig.13), alveolar bone (Fig. 16), and zone of mixed spicules (ms) of the metaphyseal surface of the proximal epiphyseal plate of the humerus (Fig. 19). All the osteoclasts show silver grains predominantly localized at their periphery. The osteoblasts (ob) and other cells are unlabeled. b, bone; bv, blood vessel.

Figures 14, 17, and 20 obtained from control animal injected with ^{125}I-sCT and an excess of cold sCT; calvaria (Fig. 14), alveolar bone (Fig.17), and zone of mixed spicules (ms) of the metaphyseal surface of the proximal plate of the humerus (Fig. 20). No silver grains are found over the osteoclasts (oc). b, bone; ob, osteoblast.

Figures 15, 18, and 21 obtained from the control animal injected with ^{125}I-sCT mixed with an excess of cold bPTH; calvaria (Fig. 15), alveolar bone (Fig. 18), and zone of mixed spicules (ms) of the metaphyseal surface of the proximal epiphyseal plate of the humerus (Fig. 20). The silver grains over the osteoclasts, have not been displaced by the excess of cold unrelated hormone demonstrating the specificity of the reaction. b, bone; ob, osteoblast.


oc
bv
13
oc
ob
14
oc
ob
15
b
b
oc
16
oc
17
oc
18
ob
oc
19
oc
20
oc
21

Figures 22 and 23 Electron microscope radioautographs of portions of osteoclasts from experimental rats. There portions are away from the bone and the calcified cartilage of the mixed spicules. They contain nuclei and a variety of cell organelles. In both figures, the silver grains are related to the cytoplasmic membrane and cytoplasmic processes (cp).

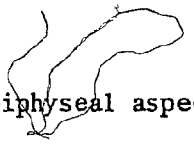
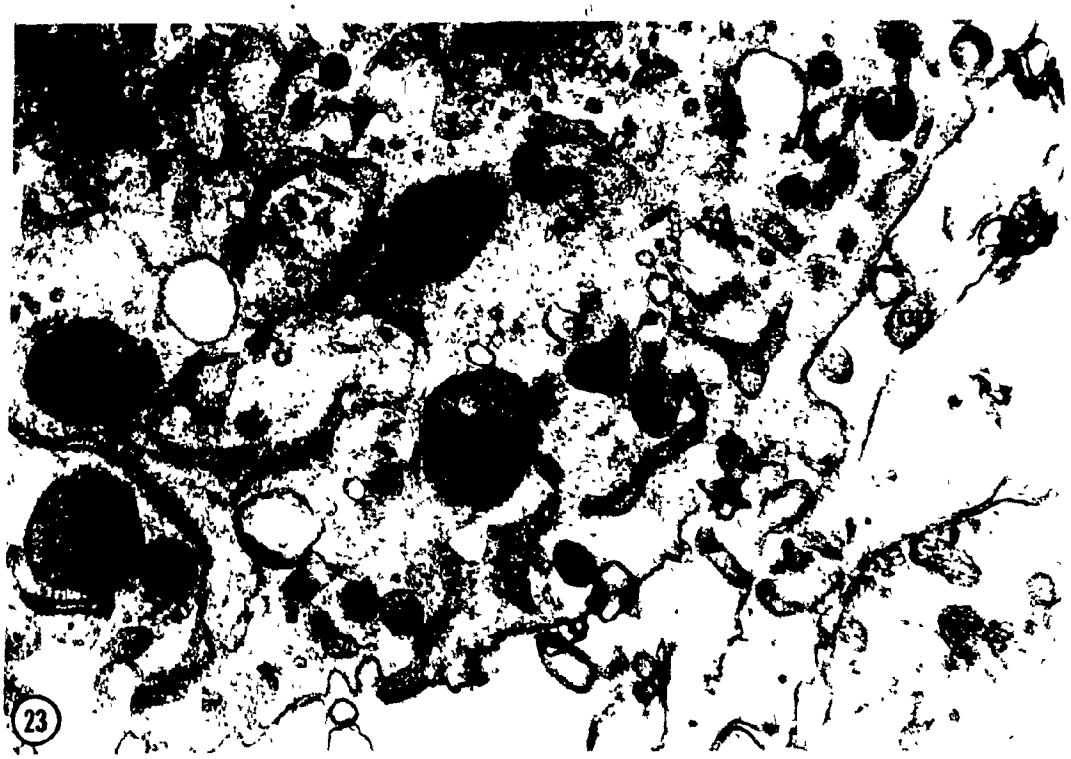


Figure 22 Osteoclast from the epiphyseal aspect of the proximal epiphyseal plate of the humerus. It contains an irregular nucleus (n), a Golgi (G), mitochondria (m), and a variety of dense bodies identify as lysosomes (L). Exposed 80 days. X 30,000.

Figure 23 Osteoclast from the epiphyseal aspect of the proximal epiphyseal plate of the tibia. The cytoplasm contains well developed Golgi (G), mitochondria (m), and a variety of dense bodies identified as primary lysosomes (L), or secondary lysosomes (sL). Exposed 80 days. X 30,000.

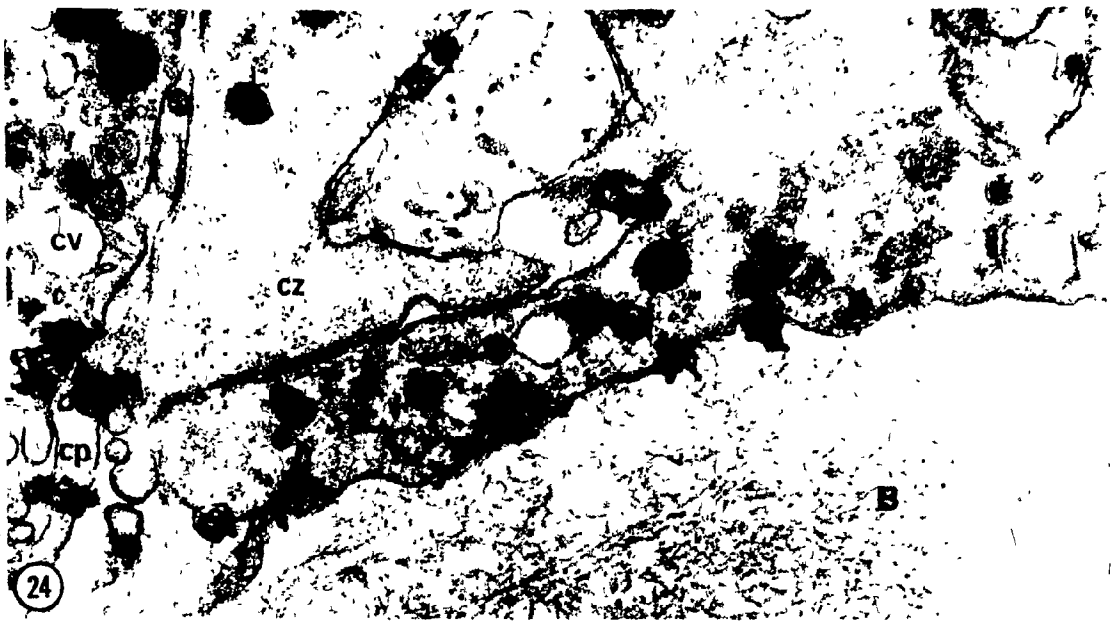

n
cp
22


23

Figures 24 and 25 Electron microscope radioautographs of portions of osteoclasts from the epiphyseal aspect of the proximal epiphyseal plate of the humerus of experimental rats showing silver grains related to the membrane over the region of vesicles and vacuoles and over the clear zone (cz).

Figure 24 Portions of adjacent osteoclasts from the epiphyseal aspect of the epiphyseal plate. These osteoclasts are covering bone (b) and contain a variety of vesicular profiles some of which are coated vesicles(cv) and dense bodies of lysosomal nature (L). The silver grains are found on the cell membrane adjacent to the bone and related to cell processes (cp). Exposed 80 days. X 30,000.

Figure 25 Most of the field is occupied by a portion of an osteoclast on a piece of calcified cartilage (CC). It contains the region of vesicles and vacuoles, mitochondria (m) and a variety of lysosomes (L). The silver grains are related to the cytoplasmic membrane adjacent to the calcified cartilage and clear zone. Exposed 52 days. X 30,000.


cv
cz
cp
B
24

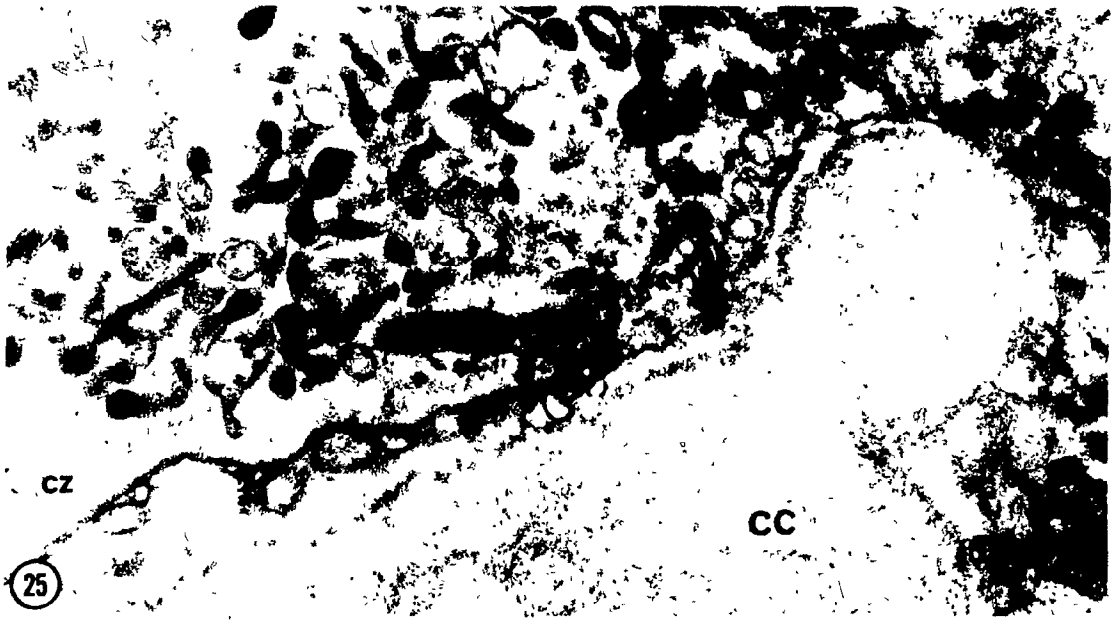

cz
CC
25

Figure 26 Electron microscope radioautograph of a portion of an osteoclast (oc) from the epiphyseal surface of the epiphyseal cartilagenous plate of the an experimental rat. This micrograph shows silver grains related to the numerous cytoplasmic cell processes (cp) and to the various vesicular profiles among which are some coated vesicles (cv). This portion of the osteoclast contains numerous mitochondria (m), and dense bodies some of which are lysosomes (L). It is closely related to a blood vessel (bv). Arrow, lamina limitans; CC, calcified cartilage; B, bone. Exposed 52 days. X 30,000.


bv
CV
CC
B
26

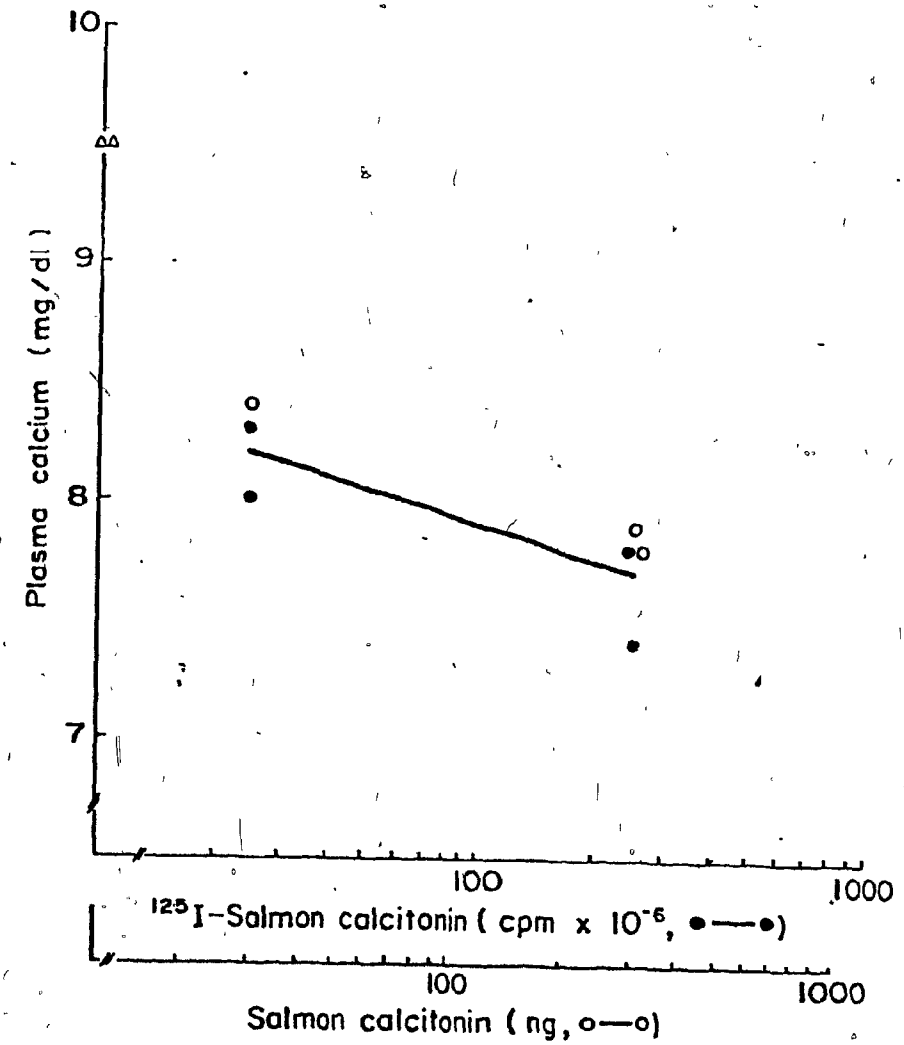
Figures 27 and 28 Light microscope radioautographs of 4 µm thick paraffin sections of the body of the subfornical organ of the brain.

Figure 27 Obtained from the experimental animal. The heavy radioautographic reaction seems to be predominantly situated around the blood vessels. Exposed 4 weeks. X 170. (*), third ventricle.

Figure 28 Obtained from the control animal. Few silver grains are present as a result of the competition between the excess cold sCT with the ^{125}I-sCT for the occupation of the specific receptor sites. Exposed four weeks. X 170. (*), third ventricle.


27
28

Figure 29. Changes in plasma calcium produced one hour after intravenous injection of ^{125}I-salmon calcitonin (●) and unlabeled salmon calcitonin (o). Basal plasma calcium (△) was determined one hour after injection of buffer only.


10
9
8
7
Plasma calcium (mg/dl)
100
1000
125I-Salmon calcitonin (cpm x 10-6, ●—●)
100
1000
Salmon calcitonin (ng, o—o)

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